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Broadly Applicable Nanowafer Drug Delivery System for Treating Eye Injuries

PRINCIPAL INVESTIGATOR: Dr. Stephen C. Pflugfelder, M.D.

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Eye injuries require immediate and effective treatment to prevent corneal opacification, neovascularization , irregularity and occasionally ulceration of the cornea, which can be potentially blinding. Eye injuries are generally treated with eye drops for 4-8 times per day, which may not be feasible in critically injured patients in intensive care. This research project aims to develop a nanowafer drug delivery system that can deliver the drug to the eye for longer periods of time to treat eye injuries and prevent potential loss of vision. During the second year of this project, the nanowafer fabrication and drug release in the eye have been optimized. The ability of the nanowafer to adherence and dissolution on the cornea has been optimized. The ability of the nanowafer to enable the drug molecules to diffuse into the corneal tissue for up to 2 days has been optimized. Presently studies are underway to evaluate the efficacy of Doxcycline, Dexamethasone, and Cyclosporine-A loaded nanowafers in ocular burn induced mouse model. These studies focus on quantification of the drug-nanowafer efficacy by time to epithelial healing using confocal fluorescence imaging and measurement of expression of relevant inflammatory mediator genes by real-time PCR.

15. SUBJECT TERMS
Nanowafer, fabrication, drug delivery, doxycycline, dexamethasone, cyclosporine-A, pharmacokinetics, ocular burn

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1. INTRODUCTION

Soldiers affected by eye injuries require immediate and effective treatment. The acute phase occurs at the time of the injury and results in corneal and conjunctival epithelial damage or necrotic death. These events lead to opacification, neovascularization, irregularity and occasionally ulceration of the cornea, which can be potentially blinding. Eye injuries are generally treated by simply introducing drug solution in the form of eye drops; however achieving sustained therapeutic levels on the ocular surface remains a challenge due to the continuous tear clearance through the lacrimal drainage system. Modulation of the ocular surface response to trauma requires multiple dosing (4-8 times per day) of the eye drops to achieve an effect, which may not be feasible in critically injured patients in intensive care. Hence, there is a strong need for the development of broadly applicable nanowafer drug delivery systems with high drug content and long term drug release attributes. This research project focuses on the development of a nanowafer drug delivery system that can deliver the drug to the eye in a controlled release fashion for longer periods of time to treat eye injuries and prevent potential loss of vision. In this project, by integrating the nanotechnologies and controlled release drug delivery technology, a nanowafer drug delivery system will be developed that can surmount the limitations of conventional eye drop formulations. The nanowafers will be fabricated *via* the hydrogel template strategy. The nanowafer contains an array of nanoreservoirs loaded with drug matrix. Upon instillation, because the nanowafer is very thin and comprised of mucoadhesive biomaterial, it readily adheres to the conjunctiva and can remain intact for several days without being displaced due to constant blinking. The nanowafer drug delivery system can release the drug in therapeutically effective concentration from a day to a week. The broadly applicable nanowafer drug delivery system upon development can be used for treating ocular surface injuries and also dry eye, corneal ulcers, glaucoma, and infections, and improve the performance efficiency and effectiveness of the soldiers in the warzone.

2. Keywords

nanowafer, nanofabrication, drug delivery, ocular burn, doxycycline, dexamethasone, cyclosporine-A, pharmacokinetics

3. ACCOMPLISHMENTS:

What were the major goals of the project?

This project focuses on accomplishing the following 5 defined tasks as proposed in the SOW:

Task 1. Regulatory approvals (IACUC/ACURO/HRPO)**.** Duration: 3 months (months 1-3)

Task 2. Fabrication of nanowafer drug delivery systems**.** Duration 18 months (months 1-18)

Task 3. Evaluation of *in vitro* and *in vivo* pharmacokinetics. Duration 24 months (months 6-30)

Task 4. Study of the efficacy of doxycycline-nanowafers, dexamethasone-nanowafers, and cyclosporine-A-nanowafers in an ocular burn mouse model. Duration: 18 months (months 18-36)

Task 5. Data Analysis**.** Duration: 6 months (months 30-36)

What was accomplished under these goals?

This section summarizes the results obtained in our laboratories during this reporting period: 1 Oct 2014 to 30 Sep 2015. Specifically, we have accomplished: the following objectives defined under each task.

Task 1. Regulatory approvals (IACUC/ACURO/HRPO)
Duration: 3 months (months 1-3)

This Task has been accomplished and reported in the Year-1 Report (09-2014)

Task 2. Fabrication of nanowafer drug delivery systems
Duration 18 months (months 1-18)

Objective (i) Nanofabrication of silicon master templates with feature dimensions of 100, 200 nm, 500 nm, 1 μm, 1.5 μm, and 3 μm

This objective has been accomplished and reported in the Year-1 Report (09-2014)

Objective (ii) Fabrication of polyvinylpyrrolidone (PVP), dextran (DTR), carboxymethyl cellulose (CMC), and hydroxypropyl cellulose (HPMC) nanowafers

This objective has been accomplished and reported in the Year-1 Report (09-2014)

Objective (iii) Fabrication of drug-filled nanowafers: doxycycline-nanowafers; dexamethasone-nanowafers; and cyclosporine-A-nanowafers

This objective has been accomplished and reported in the Year-1 Report (09-2014)

Objective (iv) Optimization of in vivo compliance after the instillation of nanowafers on cornea and conjunctiva and evaluate their adherence and dissolution by bright field and fluorescence microscopy

This project is focused on the development of ocular drug delivery nanowafer, wherein the drug and the drug carrying polymer work synergistically to provide an augmented therapeutic effect compared to conventional eye drop therapy. The nanowafer is a tiny transparent circular disc that can be simply applied on the cornea with a fingertip, like a contact lens and can withstand constant blinking without being displaced (**Figure 1A-B**). It contains arrays of drug-loaded nanoreservoirs from which the drug will be released in a tightly controlled fashion for an extended period of time (**Figure 1C-D**). The slow drug release from the nanowafer increases the drug residence time on the ocular surface and its subsequent absorption into the surrounding ocular tissue. At the end of the stipulated period of drug release, the nanowafer will dissolve and fade away. The nanowafer is highly transparent and when applied, it will have minimal effect on normal vision (**Figure 1E**).

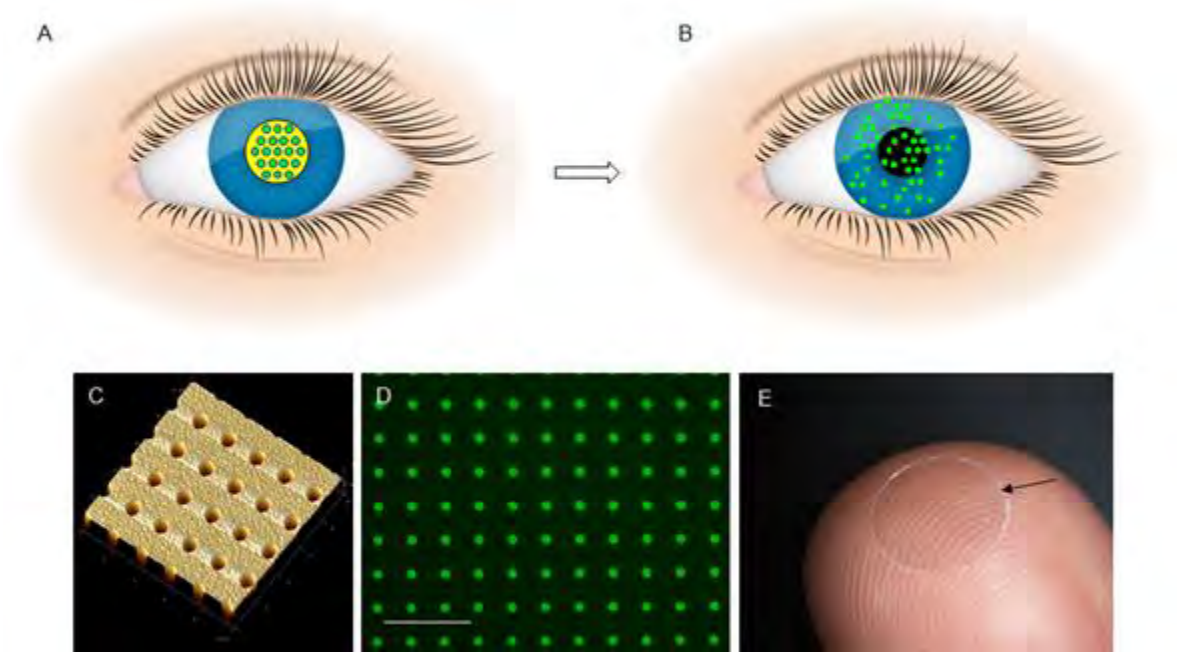

Figure. 1. Ocular drug delivery nanowafer. Schematic of (A) nanowafer instilled on the cornea and (B) diffusion of drug molecules into the corneal tissue. (C) AFM image of a nanowafer demonstrating an array of 500 nm diameter nanoreservoirs. (D) Fluorescence micrograph of a nanowafer filled with doxycycline (scale bar 5 µm). (E) Optical micrograph of a nanowafer on fingertip demonstrating the transparency of the nanowafer: the finger print lines are clearly visible through the nanowafer (arrow pointing the nanowafer).

Compliance of nanowafer on the mouse eye

To evaluate the adherence and compliance of the nanowafer on the cornea, a nanowafer was applied on the mouse cornea and examined under a stereoscopic microscope. The nanowafer cleanly adhered to the cornea in compliance with its curvature, and no wrinkling or displacement of the nanowafer was observed (**Figure 2**).

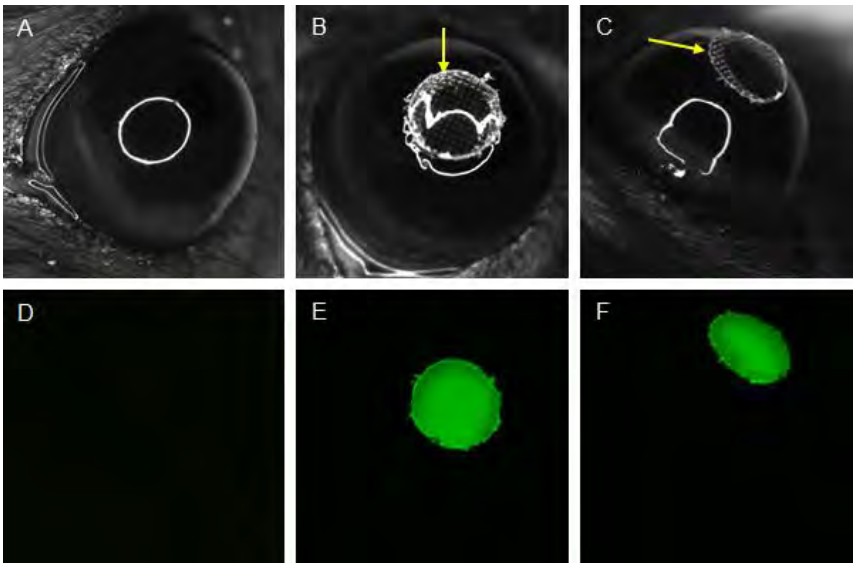

Figure 2. Demonstration of adherence and compliance of nanowafer on the mouse cornea. Optical micrographs of: (A) a mouse eye prior to nanowafer application; nanowafer applied on the mouse eye (B) top view, (C) side view. Fluorescence micrographs of: (D) a mouse eye prior to nanowafer application; doxycycline-nanowafer applied on the mouse eye (E) top view, (F) side view.

Evaluation of dissolution kinetics of the nanowafer on mouse eye

To evaluate the adherence and dissolution of a nanowafer on the cornea, a nanowafer was applied on the mouse cornea and monitored by real-time bright field and fluorescence microscopy. The nanowafer upon application, readily adhered to the cornea. The nanowafer withstood blinking and did not get displaced, thus demonstrating its mucoadhesive nature. The nanowafer dissolved and completely disappeared after 1 h (**Figure 3** Top panel). Real-time fluorescence imaging revealed that, although the nanowafer completely dissolved after 1 h, the cornea was green fluorescent for up to 5 h, confirming the diffusion of fluorescein dye into it (**Figure 3** Bottom panel).

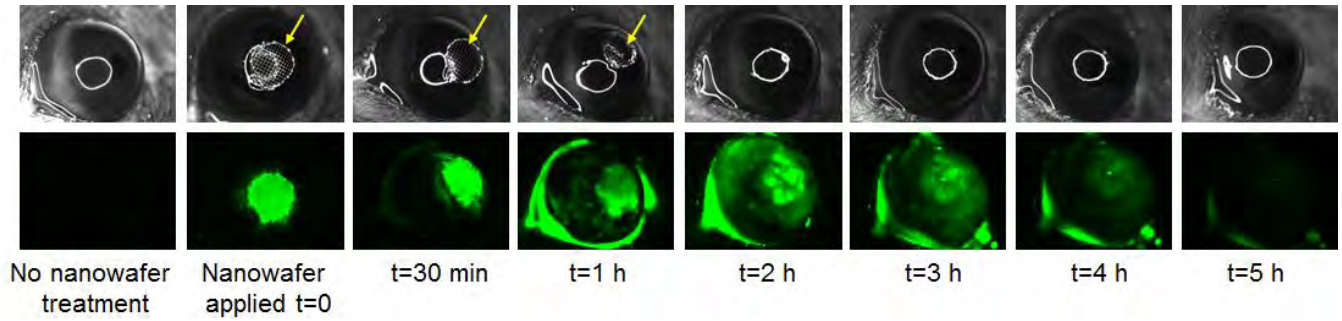

Figure. 3. Demonstration of adherence and dissolution of a nanowafer applied on the cornea. Top panel: Bright field micrographs demonstrating the adherence and dissolution of the nanowafer after 1 hour (please see the arrow pointing the position of the nanowafer). Bottom panel: Fluorescence micrographs demonstrating the presence of fluorescein in the corneal tissue for up to 5 hours.

Task 3. Evaluation of *in vitro* and *in vivo* pharmacokinetics
Duration 24 months (months 6-30)

Objective (i) Study of in vitro drug release and drug concentration in doxycycline-nanowafers, dexamethasone-nanowafers, and cyclosporine-A-nanowafers by fluorescence spectrophotometry and high performance liquid chromatography (HPLC) method

This objective has been accomplished and reported in the Year-1 Report (09-2014)

Objective (ii) Study of pharmacokinetics of doxycycline-nanowafers, dexamethasone-nanowafers, and cyclosporine-A-nanowafers in the tear washings of mice

The nanowafers were tested for their *in vivo* drug release in healthy mice. In healthy mice, the drug released can be accurately measured compared to ocular burn induced mice because, the drug will not be immediately consumed for therapeutic activity. For this study, 2 mm diameter nanowafers were used. Doxcycline loaded nanowafers (Doxy-NW) and Dexamethasone loaded nanowafers (Dex-NW) were fabricated for this study. A nanowafer was placed on the cornea and the drug release was monitored every hour by collecting the tear washings followed by HPLC analysis.

Collection of mouse tear fluid washings: A drug nanowafer was applied on the corneas of healthy mice and the tear fluid washings were collected by first instilling 1.5 μL of PBS containing 0.1% bovine serum albumin (BSA) in the conjunctival sac. The tear fluid was collected with a 1 μL volume glass capillary at hourly time intervals for 5 h. The tear washings were stored at -80 ºC until all the tear samples were collected. The drug concentration in tear washings was determined by HPLC method.

Drug release from the nanowafers in mouse tear samples

To monitor the drug concentration on the ocular surface as a measure of drug release from the nanowafer, tear samples were collected hourly for 5 h, and the doxycycline content was analyzed by HPLC. After placement of a doxycycline nanowafer on the cornea, the drug concentration in the tears slowly decreased with time, and after 4 h, no detectable doxycycline concentration was present (**Figure 4**).

To assess the drug release from the Dex-NW after its instillation on the conjunctiva, tear samples were collected at hourly intervals and analyzed for Dex concentration by HPLC. This study revealed the presence of Dex in tear samples for up to 2 h, at significantly greater concentrations than eyes treated with Dex eye drops containing the same amount (10 μg in 2 μl) of the drug (**Figure 5**). Also, in the case of Dex release from the nanowafer, more drug was present in the first hour tear samples compared to the second hour samples. Because of the ocular surface barriers, such as reflex tearing and tight epithelial junctions, the drug diffusion into the cornea is very slow in the beginning, hence more drug was present in the first hour tear samples. However, with longer drug residence time provided by the nanowafer, more drug penetrates into the cornea. This results in a decrease in drug concentration in the tear samples collected at later time points.

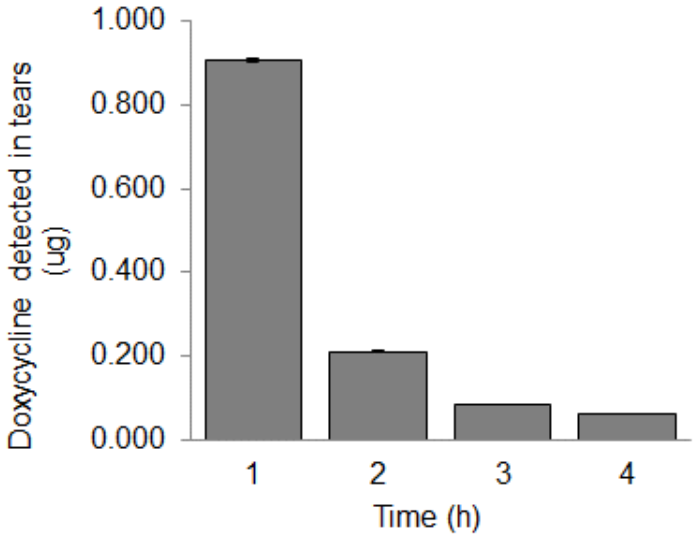

Figure 4. In vivo drug release from the Doxy-NW on the ocular surface. Doxy concentration measured in tear samples collected from eyes treated with Doxy-NW.

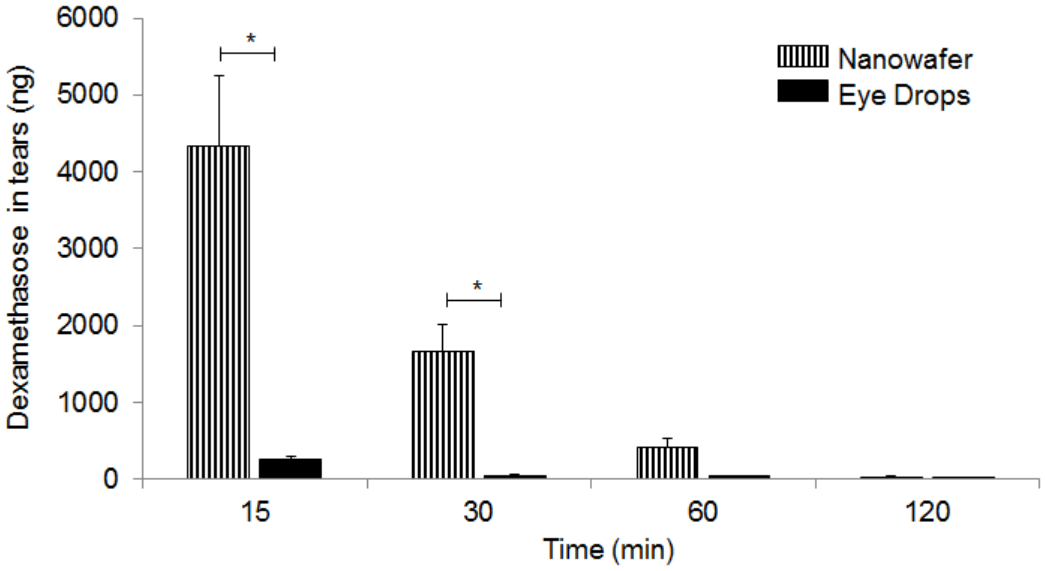

Figure 5. In vivo drug release from Dex nanowafer on the ocular surface.
Dex concentration measured in tear samples collected from eyes treated with Dex-NW or Dex eye drops.

Objective (iii) Study of in vivo pharmacokinetics after the instillation of drug-nanowafers on the cornea by real-time drug molecular transport, distribution, and residence times in the cornea and conjunctiva by laser scanning confocal fluorescence image analysis in mice for 1-10 days

To study the ability of the nanowafer in improving the drug molecular residence time on the ocular surface and its subsequent diffusion into it, fluorescein (a green fluorescent dye, MW = 332 g/mol) loaded nanowafers (Flo-NW) were fabricated. The Flo-NWs were instilled on the corneas of healthy mice and were examined by fluorescence imaging at every hour for 5 h. The ocular surface was initially non-fluorescent (**Figure 6A**). After the placement of a nanowafer, the fluorescein molecules were slowly released on the cornea and green fluorescence was observed. Since, the fluorescein released from the nanowafer is in direct contact with the cornea, most of the drug will be able to penetrate into it. After 4 h, the green fluorescence of the cornea started to fade because of the diffusion of fluorescein molecules into the anterior chamber and its clearance by tear secretion (**Figure 6B-G**). Once the fluorescein molecules pass through the cornea and reach the aqueous humor in the anterior chamber, they are cleared through the trabecular meshwork.

In comparison, eyes treated with fluorescein eye drops were non-fluorescent after 5 min, indicating its rapid clearance from the ocular surface (**Figure 6I-L**). Also, after instillation of the fluorescein drops on the eye, within a few minutes, most of it is concentrated on the eye lids, indicating its clearance from the ocular surface, compared to the nanowafer drug release, wherein only a small amount of fluorescein concentrated around the eyelids and most of it in the eye. This study has qualitatively demonstrated the ability of nanowafer to release the drug for a few hours thus improving the drug residence time on the cornea. To quantify the amount fluorescein present at each time point, the fluorescence intensities were plotted in **Figure 7**.

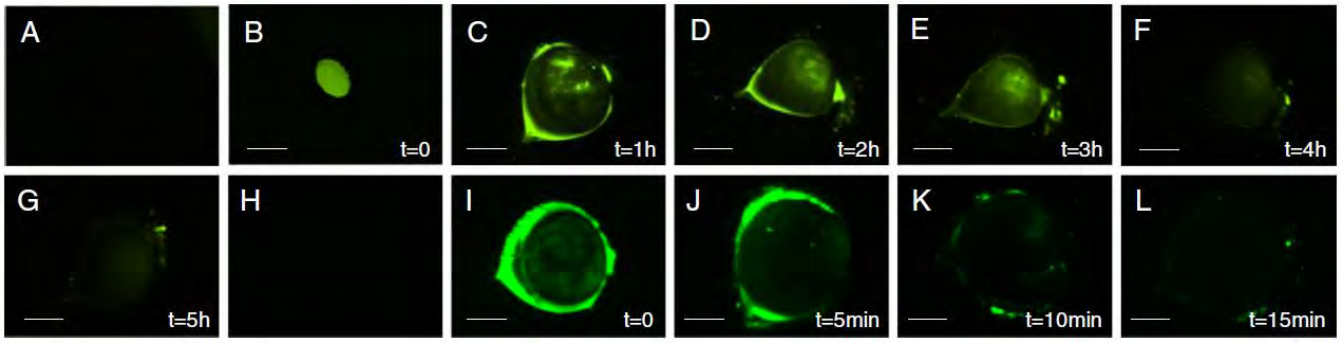

Figure 6. Nanowafer improves the drug residence time on the cornea. (A) Fluorescence micrograph of the eye prior to fluorescein nanowafer (Flo-NW) instillation; (B–G) fluorescence micrographs depicting the presence of fluorescein dye at the specified time points in the corneal tissue after Flo-NW instillation. (H) Fluorescence micrograph of the eye prior to fluorescein eye drops instillation; (I-L) Rapid clearance of fluorescein dye drops within 5 min.

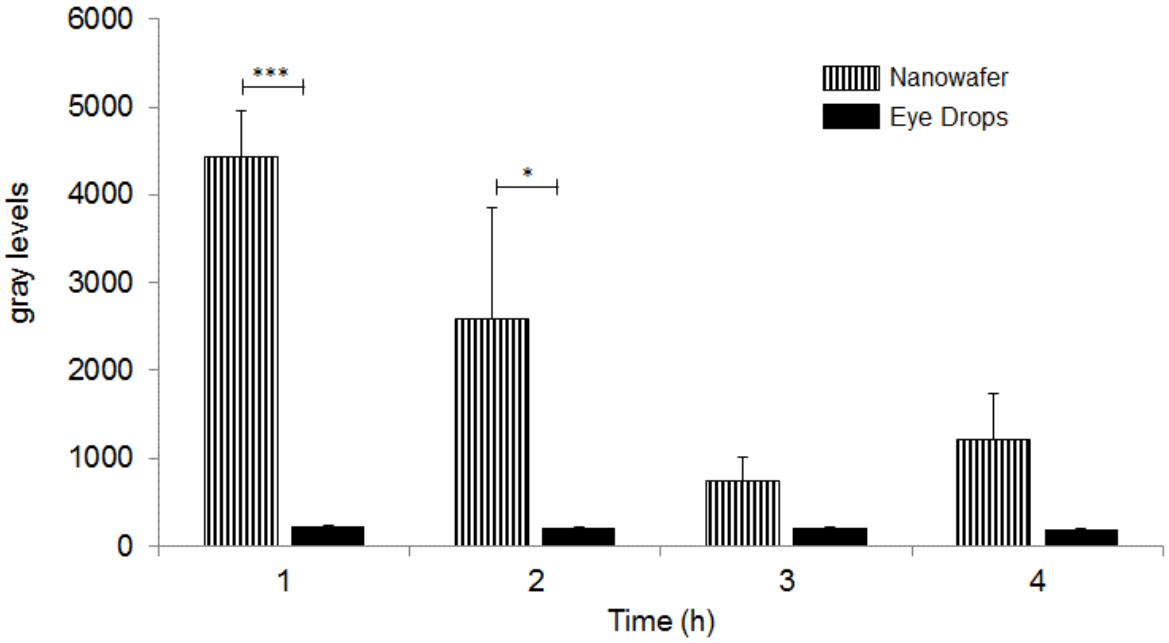

Figure. 7. Nanowafer improves the drug residence time on the cornea. A plot depicting the fluorescence intensities (n=3).*P <0.05, ***P <0.001. All error bars represent standard error of the mean. Scale bar: 1 mm

Nanowafer Enhances Drug Diffusion into the Cornea.

To demonstrate the ability of the nanowafers to release the drug for an extended period of time, PVA nanowafers loaded with doxycycline were fabricated. Doxycycline (antibiotic drug) was chosen for this study because of its green fluorescence, which allowed us to monitor the precorneal drug residence time and its subsequent diffusion into the cornea by real-time fluorescence imaging.

To monitor the drug diffusion into the mouse cornea a doxycycline nanowafer was applied on a mouse cornea (**Figure 8A**). The mouse eye was subjected to fluorescence imaging at hourly intervals while the mouse was under general anesthesia. The corneas were green fluorescent even after 5 h when viewed under a stereomicroscope with 488 nm illumination, indicating the presence of drug in the cornea (**Figure 8B-F**). The corneas exhibited a strong green fluorescence for up to 48 h, when subjected to intravital laser scanning confocal imaging, indicating the presence of doxycycline in the corneal tissue (**Figure 8G-H**). To compare the efficacy of the doxycycline delivery by nanowafer with topical eye drop treatment, another group of mice were treated with doxycycline eye drops. Upon examination under a fluorescence microscope, the corneas did not exhibit a measurable green fluorescence, indicating the complete clearance of the drug within 15 minutes (**Figure 8I-J**).

Although, doxycycline concentration was undetectable in tears after 4 h (**Figure 4**), the fluorescence and intravital confocal imaging studies have confirmed the presence of the drug in the corneal tissue for up to 48 h. Taken together, this study clearly demonstrated the ability of the nanowafer to release doxycycline for an extended period of time, thus enhancing the precorneal drug residence time and subsequent diffusion of drug molecules into the cornea.

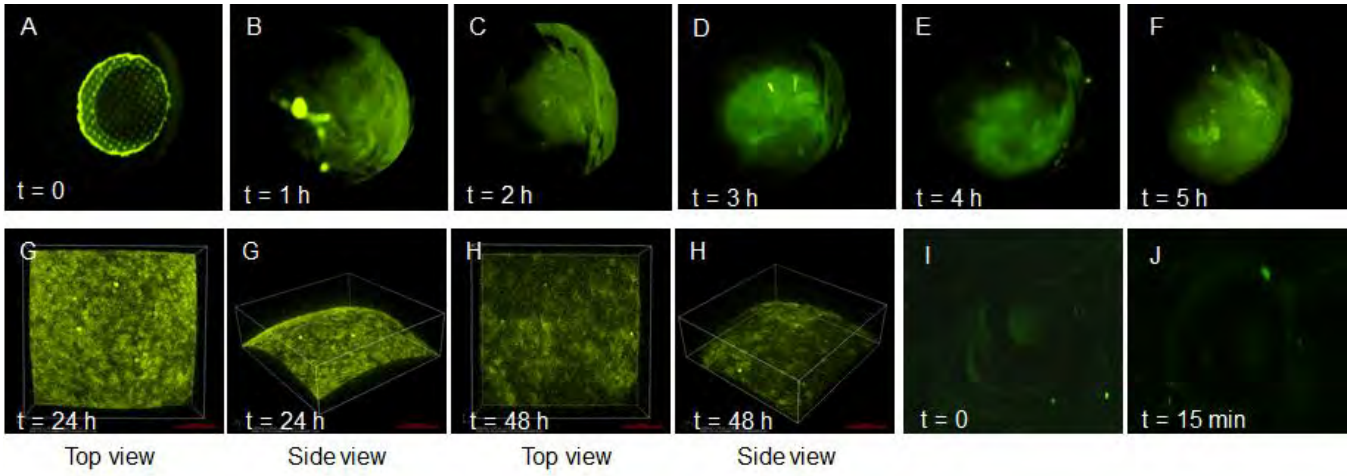

Figure 8. Nanowafer drug delivery enhances drug molecular transport into the cornea. (A-F) Real-time in vivo fluorescence imaging of the mouse cornea at regular time intervals demonstrating the doxycycline release from the nanowafer into the cornea. (G-H) Intravital confocal fluorescence image of the mouse eye showing the presence of doxycycline for up to 48 hours. (I-J) Rapid clearance of doxycycline eye drops in 15 min in mice.

Task 4. Study of the efficacy of doxycycline-nanowafers, dexamethasone-nanowafers, and cyclosporine-A-nanowafers in ocular burn mouse model

Duration: 18 months (months 18-36)

Objective (i) Quantification of the drug-nanowafer efficacy by time to epithelial healing Using confocal fluorescence imaging

This study is presently in progress

Objective (ii) Measurement of expression of relevant inflammatory mediator genes by real-time PCR

Immunostimulatory Properties of Nanowafers.

The inflammatory response to injury is important to recovery, but if dysregulated can enhance tissue damage, stimulate angiogenesis, disrupt healing, and cause corneal opacity. Since the cornea is optically clear and avascular, its neovascularization and opacification result in eventual loss of vision. The biopolymers used in the nanowafer fabrication is crucial, as they can be immunostimulatory, i.e., can induce inflammation and exacerbate the condition, leading to delayed wound healing and incomplete recovery. Choosing the right biopolymer that is nonstimulatory to the cornea's immunological and inflammatory responses and that improves the tolerability, stability, and therapeutic effect of the drug with minimal side effects is vital for the success of the ocular drug delivery nanowafer.

As a first step, a quantitative analysis of the ability of the polymers to elicit proinflammatory and proangiogenic responses in an ocular burn (OB) induced mouse model was developed. The polymer nanowafers (tiny circular discs of 2 mm diameter and 100 μm thickness) were instilled on the corneas of OB-induced mice, daily for 5 days. At the end of the treatment period, the corneas were collected and processed for evaluating proinflammatory and proangiogenic genes by reverse transcription polymerase chain reaction (RT-PCR) analysis.

During the wound-healing process, the expression levels of several proinflammatory interleukins, IL-1R, IL-1β, and IL-6, tumor necrosis factor TNF-R, and proangiogenic matrix metalloproteinases MMP-2, MMP-8, MMP-9, and MMP-13, and transforming growth factor TGF-β1 will be upregulated. Quantification of the expression levels of these factors gives insights into the effect of polymer materials on inflammation and angiogenesis. In this study, proinflammatory and proangiogenic attributes of Poly(vinyl alcohol) (PVA), Polyvinylpyrrolidone (PVP), (Hydroxypropyl)methyl cellulose (HPMC), and Carboxymethyl cellulose (CMC) nanowafers were evaluated in the corneas after injury by RT-PCR analysis (**Figure 9**). The PVA nanowafers were nonstimulatory, and the expression levels of proinflammatory factors were almost equal to the OB control group, while the PVP, HPMC, and CMC nanowafers significantly upregulated the expression of one or more inflammatory cytokines (IL-1R, IL-1β, and TNF-R) compared to the OB control group. The PVP, HPMC, and CMC nanowafers stimulated the expression levels of MMP-8, MMP-9, and TGF-β1, while the PVA nanowafers were nonstimulatory, and the expression levels of these factors were very close to the OB control group.

The polymer nanowafers were further investigated for their effect on the expression levels of proangiogenic vascular endothelial growth factor A (VEGF-A), tyrosine kinase receptors VEGF-R1 and VEGF-R2, and platelet-derived growth factor receptors (PDGFR-A and PDGFR-B). The PVA nanowafer has a nonstimulatory effect on the expression levels of these factors, and they remain very close to the OB control group. However, all other polymer nanowafers have upregulated VEGF-R1, VEGF-R2, and PDGFR-B expression levels.

Presently, PCR studies are underway to evaluate the efficacy of Dexamethasone-Nanowafers, Doxycycline-Nanowafers, and Cyclosporin-A-Nanowafers in controlling inflammation and wound healing in ocular burn induced mouse model.

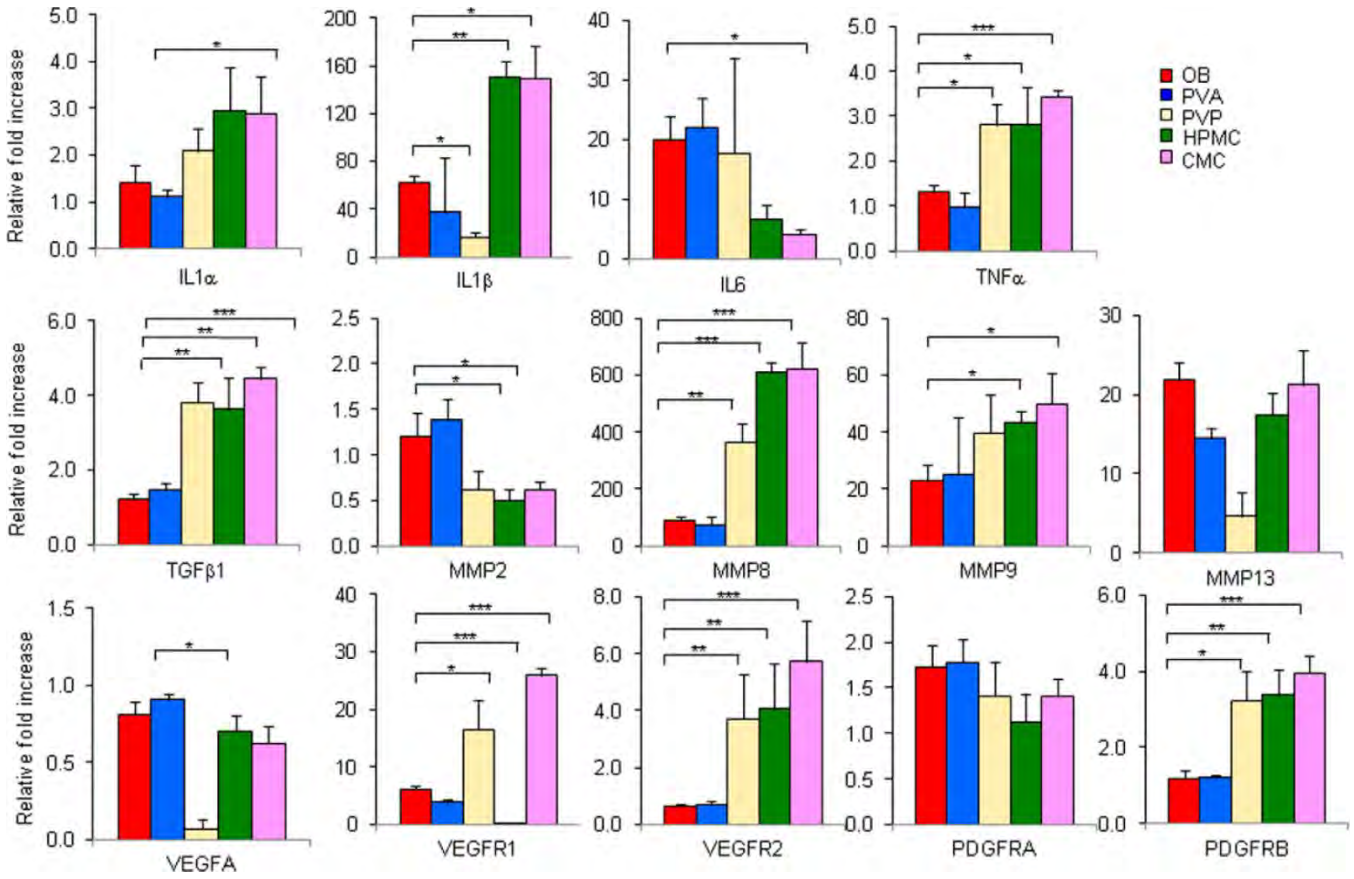

Figure 9. Analysis of inflammatory properties of biopolymers used in the nanwoafer fabrication. RT-PCR analysis confirming that PVA nanowafers do not induce upregulation of pro-inflammatory and proangiogenic factors. n = 3 (5 animals per group), *P < 0.05, **P < 0.01, ***P < 0.001). All error bars represent standard deviation from the mean.

What opportunities for training and professional development has the project provided?

1. **Dr. Daniela Marcano, Ph.D.** a postdoctoral research associate working on this project was provided necessary training and professional development opportunities. Specifically, Dr. Marcano was trained: (i) in the fabrication of nanowafer drug delivery systems, (ii) use of Nikon laser confocal fluorescence microscope and image analysis, (iii) pharmacokinetic analysis by HPLC, and (iv) preparation of animal protocols. As part of professional development, Dr. Marcano actively participated in the lab meetings, attended BCM seminars, and ARVO meeting held in Denver, CO. In addition, during one-on-one meetings, we have systematically reviewed and analyzed Dr. Marcano's experimental protocols and results. All these activities have helped Dr. Marcano accomplish the defined objectives of the project and develop into a trained scientist.

2. **Dr. Crystal S. Shin, Ph.D.** a postdoctoral research associate working on this project was provided necessary training and professional development opportunities. Specifically, Dr. Shin was trained: (i) in the fabrication and optimization of nanowafer drug delivery systems, (ii) use of Nikon laser confocal fluorescence microscope and image analysis, (iii) in vitro pharmacokinetic analysis by HPLC. For professional development, Dr. Shin actively participated in the lab meetings, attended seminars and symposiums hosted by BCM, Rice University, and Texas Medical Center, and attended ARVO meeting held in Denver, CO. During one-on-one meetings, we have reviewed and analyzed Dr. Shin's experimental progress and results. With all these activities Dr. Shin was able to achieve the defined objectives of the project and develop into an independent scientist.

How were the results disseminated to communities of interest?
"Nothing to Report"

What do you plan to do during the next reporting period to accomplish the goals?
For the next reporting period (1 Oct 2015 to 30 Sep 2016), we plan to work on the following Tasks defined in the SOW: **Task 3:** Evaluation of *in vitro* and *in vivo* pharmacokinetics; and **Task 4:** Study of the efficacy of doxycycline-nanowafers, dexamethasone-nanowafers, and cyclosporine-A-nanowafers in an ocular burn mouse model.

4. **IMPACT**

What was the impact on the development of the principal discipline(s) of the project?
"Nothing to Report"

What was the impact on other disciplines?
"Nothing to Report"

What was the impact on technology transfer?
"Nothing to Report"

What was the impact on society beyond science and technology?
"Nothing to Report"

5. **CHANGES/PROBLEMS:**
"Nothing to Report"

6. **PRODUCTS:**

Journal publications.

1. X. Yuan, D. C. Marcano, C. S. Shin, X. Hua, L. C. Isenhart, S. C. Pflugfelder, G. Acharya, Ocular drug delivery nanowafer with enhanced therapeutic efficacy, *ACS Nano* **9**, 1749–1758 (2015).
2. T. G. Coursey, J. T. Henriksson, D. C. Marcano, C. S. Shin, L. C. Isenhart, F. Ahmad, C. S. De Paiva, S. C. Pflugfelder, G. Acharya, Dexamethasone nanowafer as an effective therapy for dry eye disease, *J. Control Release*. **213**, 168–174 (2015).

Conference papers

1. G. Acharya; X. Yuan; D. Marcano; C. Shin; X. Hua; L. Isenhart; S.C. Pflugfelder. Nanowafer Drug Delivery to Treat Corneal Neovascularization. *Invest. Ophthalmol. Vis. Sci.* 2015; 56(7):5032
2. C.S. Shin; D.C. Marcano; J.T. Henriksson; G. Acharya; S.C. Pflugfelder. Nanowafer Drug Delivery for Restoration of Healthy Ocular Surface in Dry Eye Condition. *Invest. Ophthalmol. Vis. Sci.* 2015; 56(7):321.

Presentations

1. Ghanashyam Acharya: Invited seminar: “Ocular Drug Delivery Nanowafer” at Texas Tech University, Lubbock, TX. Date: 10-20-2014.
2. Ghanashyam Acharya: Invited seminar: “Ocular Drug Delivery Nanowafer: Design and Development,” Bench to Bedside Symposium, Gavin Herbert Eye Institute, University of California, Irvine, CA. Date: 06-19-2015

Press Releases of Nanowafer publication

1. American Chemical Society Press release: “An end to the medicine dropper for eye injuries” 02-04-2015
   http://www.acs.org/content/acs/en/pressroom/presspacs/2015/acs-presspac-february-4-2015/an-end-to-the-medicine-dropper-for-eye-injuries.html

2. Chemical & Engineering News: “Dissolving Disks Deliver Drugs To The Eye” 02-05-2015
   http://cen.acs.org/articles/93/web/2015/02/Dissolving-Disks-Deliver-Drugs-Eye.html

3. National Public Radio (NPR): “Dissolving Contact Lenses Could Make Eye Drops Disappear” 02-20-2015
   http://www.npr.org/sections/health-shots/2015/02/20/387301576/dissolving-contact-lenses-could-make-eye-drops-disappear

Books or other non-periodical, one-time publications.
"Nothing to Report"

Website(s) or other Internet site(s)
"Nothing to Report"
Technologies or techniques
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Inventions, patent applications, and/or licenses
"Nothing to Report"
Other Products
"Nothing to Report"

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Name	**Stephen C. Pflugfelder, M.D.**
Project Role	Principal Investigator
Researcher Identifier	
Nearest person month worked	1
Contribution to the Project	Directed and oversaw the project performance of all experiments defined under Tasks 1, 2, and 3. Reviewed and analyzed the experimental results. Reviewed the animal protocol for IACUC/ACURO approval
Funding Support	(Complete only if the funding support is provided from other than this award).
Name	**Ghanashyam Acharya, Ph.D.**
Project Role	Co-Principal Investigator
Researcher Identifier	
Nearest person month worked	4
Contribution to the Project	Directed Tasks 2 & 3. Fabricated the silicon wafer master templates, by e-beam lithography and photolithography. Fabricated PDMS imprints. Fabricated doxycycline and dexamethasone nanowafers. Developed HPLC methods for in vitro and in vivo drug release study of doxycycline and dexamethasone from the nanowafers. Designed the experiments, reviewed and analyzed the experimental results.
Funding Support	Cystinosis Research Foundation, DOD
Name	**Daniela Marcano, Ph.D.**
Project Role	Postdoctoral Research Associate
Researcher Identifier	
Nearest person month worked	12
Contribution to the Project	Prepared the animal protocol for IACUC/ACURO submission. Fabricated the PDMS imprints. Fabricated doxycycline and dexamethasone loaded nanowafers. Performed in vitro drug release study of the nanowafers. Performed the fluorescence confocal imaging to monitor the in vivo doxycycline release in mouse eye.
Funding Support	
Name	**Crystal S. Shin, Ph.D.**
Project Role	Postdoctoral Research Associate
Researcher Identifier	
Nearest person month worked	6
Contribution to the Project	Performed and optimized the nanowafer compliance experiments on mouse eyes. Performed in vivo drug release experiments.
Funding Support	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
"Nothing to Report"

What other organizations were involved as partners?
"Nothing to Report"

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
"Not Applicable"

9. APPENDICES

Appendix 1: Journal publication
Yuan et al, Ocular drug delivery nanowafer with enhanced therapeutic efficacy, *ACS Nano* **9**, 1749–1758 (2015).

Appendix 2: Journal publication
Coursey et al, Dexamethasone nanowafer as an effective therapy for dry eye disease, *J. Control Release*. **213**, 168–174 (2015).

Appendix 3: Conference paper
Acharya et al, Nanowafer Drug Delivery to Treat Corneal Neovascularization. *Invest. Ophthalmol. Vis. Sci.* 2015; 56(7):5032

Appendix 4: Conference paper
Shin et al, Nanowafer Drug Delivery for Restoration of Healthy Ocular Surface in Dry Eye Condition. *Invest. Ophthalmol. Vis. Sci.* 2015; 56(7):321.

Appendix 1: Journal publication

Yuan et al, Ocular drug delivery nanowafer with enhanced therapeutic efficacy, *ACS Nano* **9**, 1749–1758 (2015).

Ocular Drug Delivery Nanowafer with Enhanced Therapeutic Efficacy

Xiaoyong Yuan,[†] Daniela C. Marcano, Crystal S. Shin, Xia Hua,[†] Lucas C. Isenhart, Stephen C. Pflugfelder,[*] and Ghanashyam Acharya[*]

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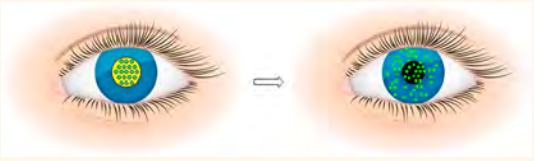
ABSTRACT Presently, eye injuries are treated by topical eye drop therapy. Because of the ocular surface barriers, topical eye drops must be applied several times in a day, causing side effects such as glaucoma, cataract, and poor patient compliance. This article presents the development of a nanowafer drug delivery system in which the polymer and the drug work synergistically to elicit an enhanced therapeutic efficacy with negligible adverse immune responses. The nanowafer is a small transparent circular disc that contains arrays of drug-loaded nanoreservoirs. The slow drug release from the nanowafer increases the drug residence time on the ocular surface and its subsequent absorption into the surrounding ocular tissue. At the end of the stipulated period of drug release, the nanowafer will dissolve and fade away. The *in vivo* efficacy of the axitinib-loaded nanowafer was demonstrated in treating corneal neovascularization (CNV) in a murine ocular burn model. The laser scanning confocal imaging and RT-PCR study revealed that once a day administered axitinib nanowafer was therapeutically twice as effective, compared to axitinib delivered twice a day by topical eye drop therapy. The axitinib nanowafer is nontoxic and did not affect the wound healing and epithelial recovery of the ocular burn induced corneas. These results confirmed that drug release from the axitinib nanowafer is more effective in inhibiting CNV compared to the topical eye drop treatment even at a lower dosing frequency.



KEYWORDS: nanowafer · drug delivery · inflammation · corneal neovascularization · therapeutic efficacy

Eye injuries are one of the major causes of blindness worldwide, and in the United States alone 2.5 million eye injuries occur every year.[1] Ocular surface injuries disrupt corneal angiogenic privilege and trigger corneal neovascularization (CNV), eventually leading to loss of vision.[2] Ocular drug delivery, although it may seem to be deceptively simple, is a challenging task mainly because of the unique barriers associated with the ocular surface that impede adequate drug delivery and therapeutic efficacy.[3] Topical drug therapy with eye drop formulations is the most accessible and noninvasive. However, its potential is limited by the ocular surface protective barriers, such as reflex tearing, constant blinking, impervious nature of the ocular surface due to tight epithelial junctions, and nasolacrimal drainage that can rapidly clear the eye drops from the ocular surface in a few minutes.[4,5] In addition, drug clearance due to systemic absorption through blood capillaries in the conjunctival sac can further reduce the amount of drug available for effective ocular absorption.[6,7] These physiological barriers contribute to inadequate drug delivery and reduced bioavailability of the drug to the eye. Hence, topical eye drops must be applied several times a day, thus increasing the potential for toxic side effects such as cellular damage, inflammation of the ocular surface, and temporary blurred vision, leading to discomfort and poor patient compliance.[8,9] Topical eye drop therapies that target CNV often produce side effects such glaucoma and cataract formation.[10,11]

To improve treatment efficacy, emulsions, liposomes, micelles, and nanoparticle suspensions have been used in ocular drug delivery. However, these formulations were also rapidly cleared from the eye.[12−15] *In situ* gel-forming systems have been developed for ocular drug delivery.[16] A hydrogel-forming solution containing drug upon instillation as eye drops undergoes sol-to-gel phase transition on the eye surface. The *in situ*-formed gels are expected to hold the drug for a longer period of time, thus enhancing its bioavailability. However, these formulations are also quickly cleared

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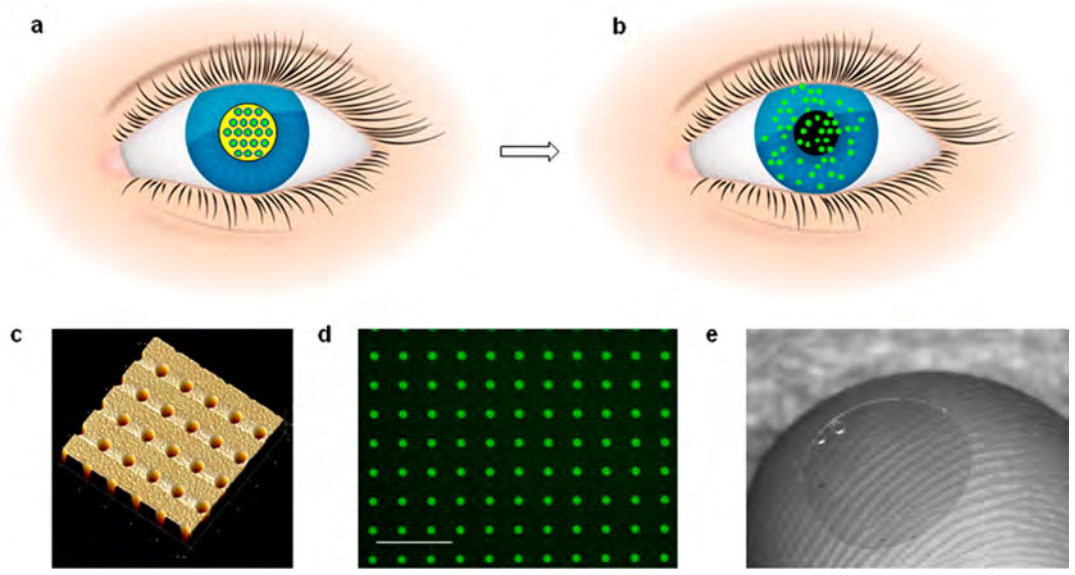

Figure 1. Ocular drug delivery nanowafer. (a) Schematic of the nanowafer instilled on the cornea. (b) Diffusion of drug molecules into the corneal tissue. (c) AFM image of a nanowafer demonstrating an array of 500 nm diameter nanoreservoirs. (d) Fluorescence micrograph of a nanowafer filled with doxycycline (scale bar 5 μm). (e) Nanowafer on a fingertip.

from the ocular surface, resulting in limited therapeutic efficacy. Drug-loaded contact lenses have been developed to improve the drug retention time in the eye.[17,18] All these systems could not release the drug for extended periods of time, leading to limited drug efficacy, thus requiring multiple administrations.

In this context, development of a novel nano drug delivery system that can surmount the ocular surface barriers and release the drug for extended periods of time, thus enhancing therapeutic efficacy and improving patient compliance, is vitally important to treat ocular injuries, infections, and inflammatory conditions and restore normal physiological functions of the eye.

RESULTS AND DISCUSSION

This article presents the development of an ocular drug delivery nanowafer, wherein the drug carrying polymer and the drug work synergistically to provide an augmented therapeutic effect compared to conventional eye drop therapy. The nanowafer is a tiny transparent circular disc that can be applied on the ocular surface with a fingertip and can withstand constant blinking without being displaced (Figure 1). It contains arrays of drug-loaded nanoreservoirs from which the drug will be released in a tightly controlled fashion for a few hours to days. The slow drug release from the nanowafer increases the drug residence time on the ocular surface and its subsequent absorption into the surrounding ocular tissue. At the end of the stipulated period of drug release, the nanowafer will dissolve and fade away. The development of an ocular drug delivery nanowafer and its enhanced *in vivo* therapeutic efficacy is herein demonstrated by treating corneal neovascularization in a murine ocular burn (OB) model.[19]

Nanowafer Fabrication. In this study, four different polymers, poly(vinyl alcohol) (PVA), polyvinylpyrrolidone (PVP), (hydroxypropyl)methyl cellulose (HPMC), and carboxymethyl cellulose (CMC), were used for nanowafer fabrication. These polymers were selected for their water solubility, biocompatibility, mucoadhesivity, transparency, and film-forming properties so as to readily adhere to a wet mucosal surface and conform to the curvature of the eye.[20] Aqueous solutions of these polymers are currently in clinical use as artificial tears, and therefore nanowafers fabricated with these polymers can function both as a drug delivery system and also as lubricant.[21,22] The nanowafers were fabricated *via* the hydrogel template strategy with a few modifications (Figure 2).[23,24] The first step involves the fabrication of arrays of wells (500 nm diameter and 500 nm depth) on a silicon wafer by e-beam lithography followed by preparation of its poly(dimethylsiloxane) (PDMS) imprint. In the second step, a polymer solution will be poured on the PDMS template followed by baking. The polymer wafer containing 500 nm diameter wells was peeled off and placed on the flat surface to expose the wells. The wells in the polymer wafer were filled with a solution of drug/polymer mixture. In this study, blank nanowafers (without the drug) of PVA, PVP, HPMC, and CMC and PVA nanowafers loaded with sunitinib, sorafenib, axitinib, and doxycycline were fabricated.

Immunostimulatory Properties of Nanowafers. The inflammatory response to injury is important to recovery, but if dysregulated can enhance tissue damage, stimulate angiogenesis, disrupt healing, and cause corneal opacity.[25,26] Since the cornea is optically clear and avascular, its neovascularization and opacification result in eventual loss of vision. The biopolymers

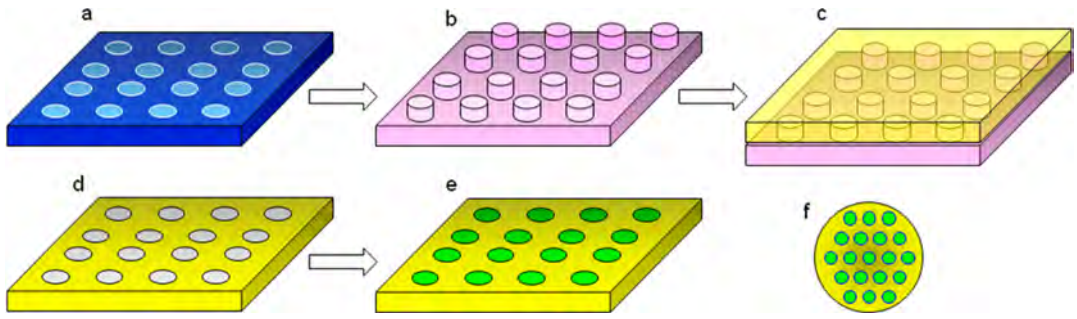

Figure 2. Schematic of nanowafer fabrication. (a) Silicon wafer master template having 500 nm wells fabricated by e-beam lithography. (b) PDMS imprint containing vertical posts. (c) Fabrication of a PVA template. (d) PVA template. (e) PVA template filled with drug. (f) Drug-filled nanowafer.

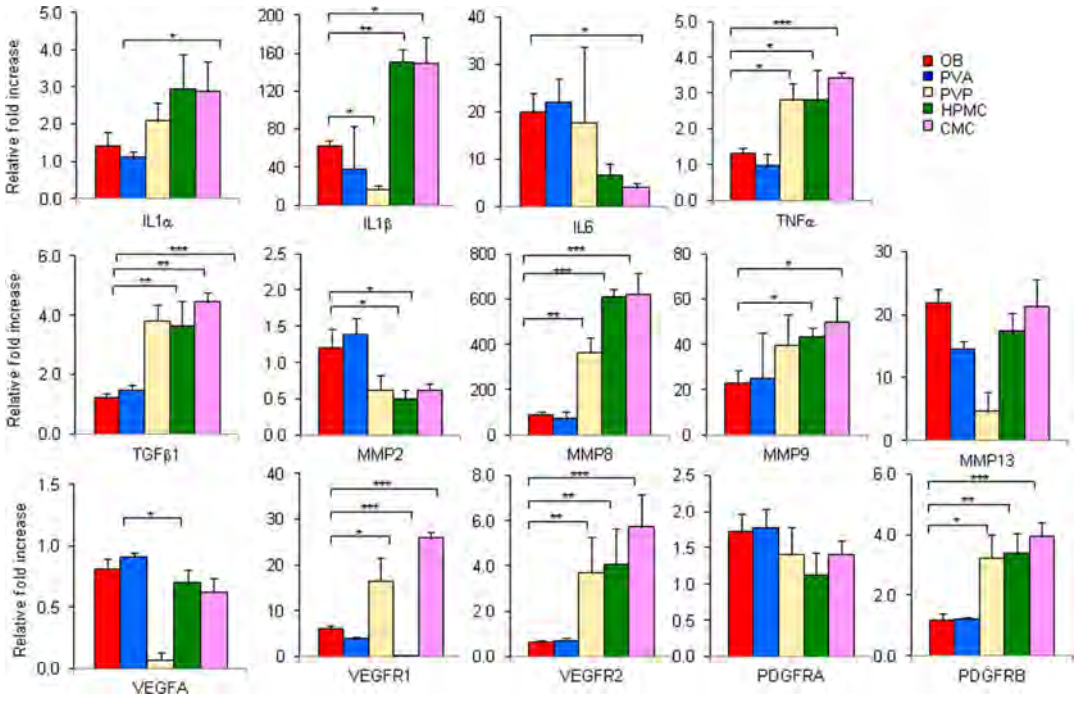

Figure 3. Poly(vinyl alcohol) nanowafer is nonimmunogenic. RT-PCR analysis confirming that PVA nanowafers do not induce upregulation of proinflammatory and proangiogenic factors. $n = 3$ (5 animals per group), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). All error bars represent standard deviation from the mean.

used in the nanowafer fabrication is crucial, as they can be immunostimulatory, *i.e.*, can induce inflammation and exacerbate the condition, leading to delayed wound healing and incomplete recovery.[27−29] Choosing the right biopolymer that is nonstimulatory to the cornea's immunological and inflammatory responses and that improves the tolerability, stability, and therapeutic effect of the drug with minimal side effects is vital for the success of the ocular drug delivery nanowafer.

As a first step, a quantitative analysis of the ability of the polymers to elicit proinflammatory and proangiogenic responses in an OB mouse model was developed. The polymer nanowafers (tiny circular discs of 2 mm diameter and 100 μm thickness) were instilled on the corneas of OB-induced mice, daily for 5 days. At the end of the treatment period, the corneas were collected and processed for evaluating proinflammatory and proangiogenic genes by reverse transcription polymerase chain reaction (RT-PCR) analysis.

During the wound-healing process, the expression levels of several proinflammatory interleukins, IL-1α, IL-1β, and IL-6, tumor necrosis factor TNF-α, and proangiogenic matrix metalloproteinases MMP-2, MMP-8, MMP-9, and MMP-13, and transforming growth factor TGF-β1 will be upregulated.[30−33] Quantification of the expression levels of these factors gives insights into the effect of polymer materials on inflammation and angiogenesis. In this study, proinflammatory and proangiogenic attributes of PVA, PVP, HPMC, and CMC nanowafers were evaluated in the corneas after injury by RT-PCR analysis (Figure 3). The PVA nanowafers were nonstimulatory, and the expression levels of proinflammatory factors were almost equal to the OB control group, while the PVP, HPMC, and CMC nanowafers significantly upregulated the expression of

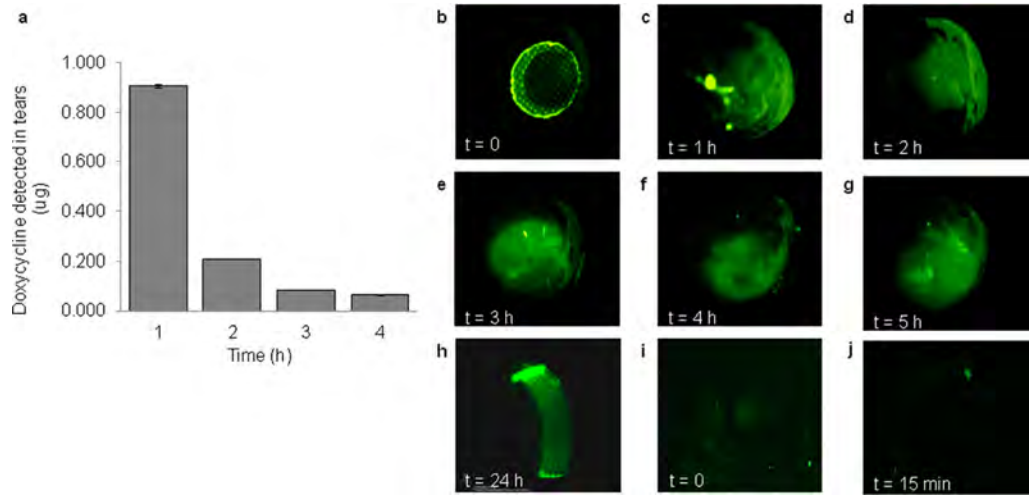

Figure 4. Nanowafer drug delivery enhances drug molecular transport into the cornea. (a) *In vivo* drug release from a doxycycline nanowafer placed on the cornea and measurement of the drug concentration in tears. (b–g) Fluorescence stereomicroscopic images of mouse cornea at regular time intervals demonstrating the doxycycline release from the nanowafer into the cornea. (h) Intravital confocal fluorescence image of the mouse eye showing the presence of doxycycline at 24 h. (i and j) Rapid clearance of doxycycline eye drops in 15 min in mice.

one or more inflammatory cytokines (IL-1α, IL-1β, and TNF-α) compared to the OB control group. The PVP, HPMC, and CMC nanowafers stimulated the expression levels of MMP-8, MMP-9, and TGF-β1, while the PVA nanowafers were nonstimulatory, and the expression levels of these factors were very close to the OB control group.

The polymer nanowafers were further investigated for their effect on the expression levels of proangiogenic vascular endothelial growth factor A (VEGF-A), tyrosine kinase receptors VEGF-R1 and VEGF-R2, and platelet-derived growth factor receptors (PDGFR-A and PDGFR-B). The PVA nanowafer has a nonstimulatory effect on the expression levels of these factors, and they remain very close to the OB control group. However, all other polymer nanowafers have upregulated VEGF-R1, VEGF-R2, and PDGFR-B expression levels. Taken together, these results indicate that PVA nanowafers are well tolerated by the ocular surface and do not stimulate the expression of proinflammatory and proangiogenic factors. To take advantage of these attributes, PVA was chosen for the fabrication of nanowafers. Furthermore, these results also indicate that the PVA nanowafers are well suited for the delivery of antiangiogenic small molecular tyrosine kinase receptor inhibitor (TKI) drugs to treat CNV.

Nanowafer Enhances Drug Diffusion into the Cornea. To demonstrate the ability of the nanowafers to release the drug for an extended period of time, PVA nanowafers loaded with doxycycline were fabricated. Doxycycline (antibiotic drug) was chosen for this study because of its green fluorescence, which allowed us to monitor the precorneal drug residence time and its subsequent diffusion into the cornea by real-time fluorescence imaging.[34] To monitor the drug concentration on the ocular surface as a measure of drug release from the nanowafer, tear samples were collected hourly for 5 h, and the doxycycline content was analyzed by HPLC.[35] After placement of a doxycycline nanowafer on the cornea, the drug concentration in the tears slowly decreased with time, and after 4 h, no detectable doxycycline concentration was present (Figure 4a). To monitor the drug diffusion into the mouse cornea after the instillation of a doxycycline nanowafer, it was subjected to fluorescence imaging at hourly intervals under general anesthesia. The corneas were green fluorescent even after 5 h when viewed under a stereomicroscope with 488 nm illumination, indicating the presence of drug in the cornea (Figure 4b–g). The corneas exhibited a strong green fluorescence even after 24 h, when subjected to intravital laser scanning confocal imaging, indicating the presence of doxycycline in the corneal tissue (Figure 4h). To compare the efficacy of the doxycycline delivery by nanowafer with topical eye drop treatment, another group of mice were treated with doxycycline eye drops. Upon examination under a fluorescence microscope, the corneas did not exhibit a measurable green fluorescence, indicating the complete clearance of the drug within a few minutes (Figure 4i and j). Although, doxycycline concentration was undetectable in tears after 4 h, the fluorescence and intravital confocal imaging studies have confirmed the presence of the drug in the corneal tissue for up to 24 h. Taken together, this study clearly demonstrates the ability of the nanowafer to release doxycycline for an extended period of time, thus enhancing the precorneal drug residence time and subsequent diffusion of drug molecules into the cornea.

Tyrosine Kinase Receptor Inhibitor Drugs to Load into the Nanowafers. TKI drugs were selected as the most suitable candidates for loading into PVA nanowafers and

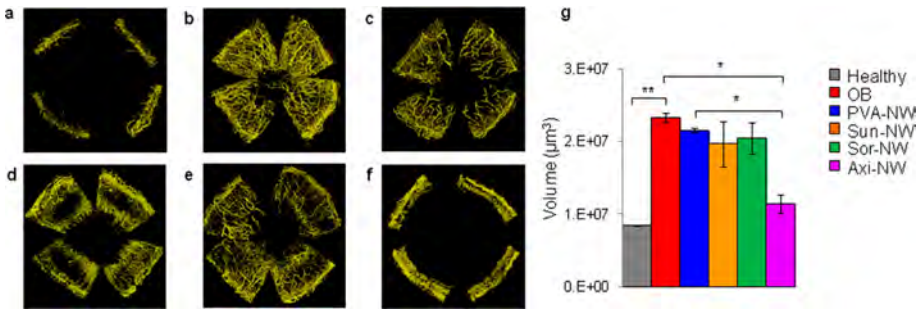

Figure 5. Selection of tyrosine kinase receptor inhibitor drugs. Screening of tyrosine kinase inhibitor drugs loaded nanowafers for their relative therapeutic efficacy in inhibiting corneal neovascularization after 10 days of treatment. Representative 3D reconstructed corneal images of fluorescence confocal microscopy: (a) healthy cornea (control); (b) untreated ocular burn (control); (c) blank PVA-NW; (d) Sora-NW; (e) Suni-NW; (f) Axi-NW. (g) Quantification of corneal neovascularization volume. $n = 3$ animals, $^{*}P < 0.05$ *vs* OB control and $P < 0.05$ *vs* PVA-NW, $^{**}P < 0.01$. All error bars represent standard deviation from the mean.

further evaluated for their relative therapeutic efficacy. The TKI drugs constitute a rapidly evolving class of low molecular weight antiangiogenic drugs that can selectively bind and inhibit the upregulation of tyrosine kinase receptors such as VEGFR1, VEGFR2, PDGFR-A, and PDGFR-B.[36] These drugs are very effective in suppression and regression of neovascularization.[37] Nanowafers containing sunitinib, sorafenib, and axitinib were fabricated for this study. These drugs were chosen, as they are already in clinical use as antiangiogenic therapeutics to treat late-stage renal carcinoma.[38−40]

To select the most suitable drug for the treatment of CNV, PVA nanowafers loaded with sunitinib (Sun-NW), sorafenib (Sor-NW), and axitinib (Axi-NW) were investigated for their relative therapeutic efficacy and compared with untreated OB group and PVA nanowafer (PVA-NW)-treated groups. For mouse experiments, each nanowafer was fabricated to be 2 mm in diameter to exactly cover the corneal surface and contained 5 μg of the drug. The nanowafers were instilled on the cornea immediately after the alkali burn. The mice were subjected to once a day nanowafer treatment for 10 days followed by a 5-day observation period. The CNV was monitored by slit-lamp imaging every other day. This study revealed an extensive neovascularization of the corneas in untreated OB and PVA-NW-treated control groups. The Sun-NW- and Sor-NW-treated groups also exhibited neovasculature. In all these groups, the new blood vessels proliferated from the limbal rim area to the center of the cornea. The Axi-NW-treated corneas were the clearest and had less CNV compared to the untreated OB control group, and the new blood vessels were closely restricted to the limbal area (Supplementary Figure S1). At the end of the treatment period, whole mount specimens of these corneas were prepared and treated with rat anti-mouse CD31 antibody and Alexa-Fluor 594-conjugated goat anti-rat secondary antibody to label vascular endothelium, and subjected to laser scanning confocal fluorescence imaging.[41] This study revealed that Sun-NW and Sora-NW were not very effective in suppressing the CNV. The Axi-NW treatment was the most effective, and the corneas appeared to be almost close to the healthy cornea with minimal CNV restricted to the limbal area (Figure 5a−f). The CNV volume was quantified for each group using the IMARIS program. The Axi-NW-treated group exhibited significantly less CNV compared to the untreated control and PVA-NW groups ($P < 0.05$). The Axi-NW is almost twice as effective as Sun-NW and Sor-NW in suppressing the CNV (Figure 5g). Taken together, these results demonstrated the pronounced therapeutic efficacy of the Axi-NW compared to the untreated control group and also the Sun-NW- and Sor-NW-treated mouse groups. On the basis of these results, Axi-NW was selected for further study.

To minimize the drug-related side effects, the maximum tolerated therapeutic dose was determined by conducting a drug escalation study. Administration of maximum tolerated dose is usually associated with maximum clinical benefit. For this study, nanowafers containing 10, 5, and 2.5 μg of axitinib (Axi-10-NW, Axi-5-NW, and Axi-2.5-NW) were fabricated and tested for their therapeutic efficacy in OB mice. Axi-5-NW and Axi-2.5-NW were the most efficacious compared to Axi-10-NW (Supplementary Figure S2). Both Axi-5-NW and Axi-2.5-NW exhibited almost the same efficacy, and the CNV volumes were significantly lower than the OB control group ($P < 0.01$). Slit-lamp examination revealed no changes in macroscopic appearance of the eyelids or the conjunctiva for 15 days during the Axi-5-NW and Axi-2.5-NW treatment and observation periods when compared with the control groups. There were no drug-related adverse effects of the Axi-5-NW and Axi-2.5-NW treatment. However, in the case of Axi-10-NW treatment, a few corneal perforations were observed after day 3. On the basis of this study, Axi-5-NW was selected as the maximum tolerated dose for comparing the efficacy of nanowafer and eye drop drug delivery methods.

Enhanced Therapeutic Efficacy of Axitinib Nanowafer. Because of the physiological barriers of the ocular surface,

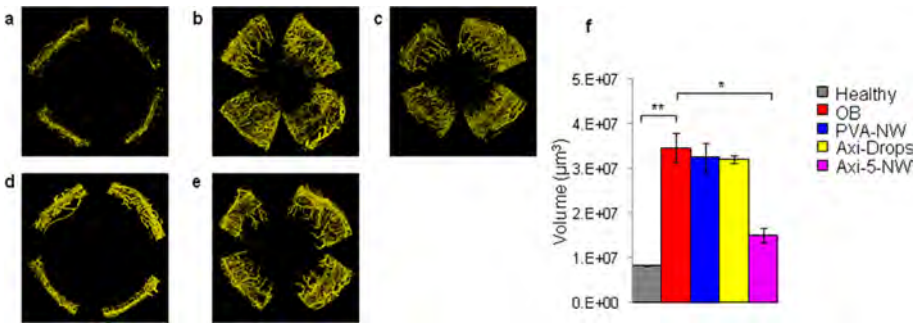

Figure 6. Axitinib nanowafer is more efficacious than the topical eye drop treatment. Representative 3D reconstructed corneal images revealing the enhanced therapeutic efficacy of Axi nanowafer compared to twice a day eye drop treatment. (a) Healthy cornea. (b) OB-induced cornea. (c) PVA-NW. (d) Axi-NW. (e) Twice a day Axi-eye drop (0.1%) treatment. (f) Quantification of corneal neovascularization volume. n = 3 animals, $*P < 0.05$ *vs* OB control. All error bars represent standard deviation from the mean.

topical eye drops must be applied several times a day for a therapeutic effect, thus increasing the potential for toxic side effects. In this study, once a day Axi-5-NW treatment was compared with Axi eye drops (0.1%) administered twice a day for its therapeutic effect in inhibiting CNV in an OB mouse model. A circular Axi-5-NW was placed on the injured cornea under general anesthesia, daily for 10 days followed by 5 days of observation. On the 15th day, the corneas were collected, processed, and subjected to laser scanning fluorescence confocal microscopy.

The images of whole mount corneas clearly demonstrated a strong therapeutic effect of the Axi-5-NW treatment compared to the untreated OB control group (Figure 6a–e). The Axi-5-NW treatment has restricted the proliferation of blood vessels to the limbal area and very closely resembled the healthy uninjured cornea. However, the OB control, PVA-NW, and Axi eye drop treated corneas exhibited an extensive neovascularization. The new blood vessels were highly branched and extended from the limbal area toward the center of the cornea. In the case of Axi-5-NW treatment, the amount of drug delivered to the cornea was 5 μg per day, and for axitinib eye drop treatment it was 10 μg per day. Although, eye drop treated mice received twice the drug dosage as those treated with Axi-5-NW, still Axi-5-NW treatment was twice as efficacious as the eye drop treatment (Figure 6f). These results confirmed that the controlled drug release from Axi-5-NW is more effective in inhibiting CNV compared to the eye drop treatment even at a lower dosing frequency.

To further compare the efficacy of Axi-5-NW and Axi eye drops at the molecular level, expression levels of the drug target factors VEGF-A, VEGF-R1, VEGF-R2, PDGFR-A, PDGFR-B, TNF-α, bFGF, and TGF-β were measured, in addition to proinflammatory interleukins and proangiogenic matrix metalloproteinases. The Axi-NW was much more effective at suppressing Axi-target genes compared to the untreated OB and Axi-eye drop treatment (Figure 7). The Axi-5-NW also showed significant suppression of proinflammatory cytokine and proangiogenic MMP expression compared to OB alone and Axi-eye drops (Supplementary Figure S3). These results reaffirmed the enhanced efficacy of axitinib when delivered by a nanowafer once a day for 10 days compared to the twice a day topical eye drop treatment for the same period of time.

Axitinib Nanowafer Improves Corneal Wound Healing. The effect of Axi-5-NW on the corneal wound healing process was studied by corneal fluorescein staining. This study revealed that the corneal wound healing was unaffected by the Axi-5-NW treatment, and a normal healing pattern was observed. The rate of epithelial closure of the corneal surface was almost the same in both Axi-5-NW and Axi-eye drop treated groups, and complete corneal surface recovery was observed by the ninth day. However, the OB control and PVA-NW-treated groups demonstrated a slightly slower recovery, and complete healing was observed by the 11th day (Figure 8). These results confirmed that axitinib is nontoxic and did not affect the epithelial recovery of the OB-induced corneas, unlike other antiproliferative agents such as mitomycin C and 5-fluorouracil, which are prone to retarding the corneal epithelial recovery.[41]

CONCLUSIONS

Ocular drug delivery systems aim to deliver the pharmacologic agent at the desired therapeutic concentration to the target ocular tissue without damaging the healthy tissue. In the treatment of ocular disease, however, this aim becomes more challenging because of the protective ocular surface barriers and highly sensitive ocular tissues. The challenge is to delicately circumvent these protective ocular surface barriers and deliver the drug to the target site (cornea or conjunctiva) without causing permanent tissue damage. Despite sustained efforts, the development and optimization of new nano drug delivery systems have been very slow. To improve the therapeutic efficacy of ophthalmic drugs, the drug delivery system

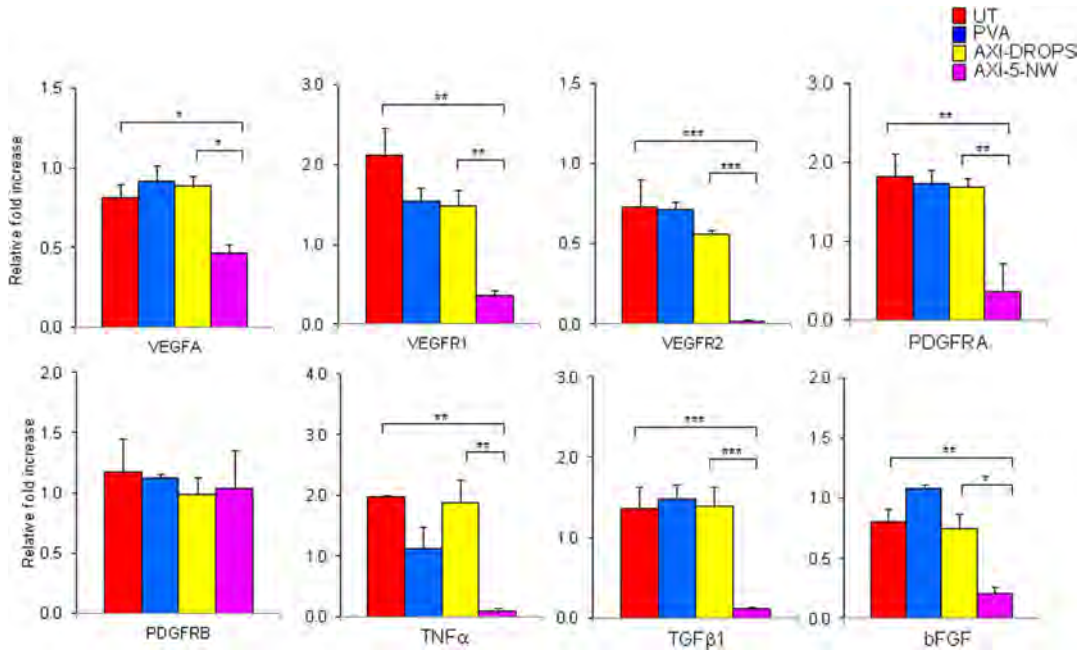

Figure 7. Enhanced therapeutic effect of axitinib nanowafer. RT-PCR analysis revealing the strong suppression of the expression levels of drug target genes by nanowafer compared to twice a day topical axitinib eye drop treatment. *n* = 3 (5 animals per group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. All error bars represent standard deviation from the mean.

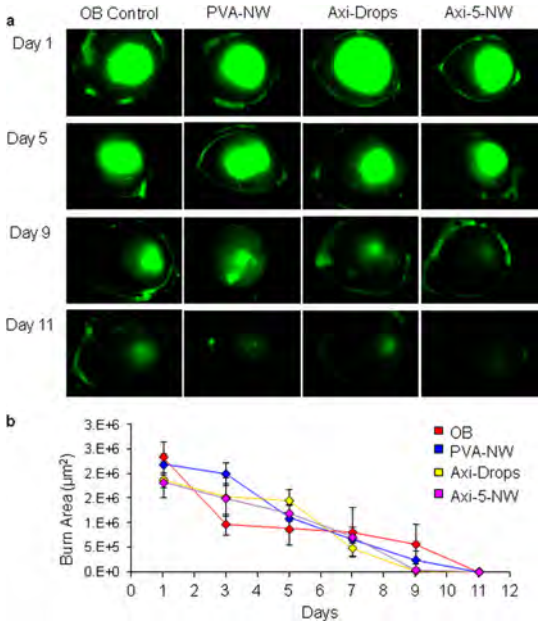

Figure 8. Axitinib nanowafer treatment improves the corneal wound healing after ocular burn injury. (a) Fluorescence images. (b) Plot demonstrating corneal surface healing as a measure fluorescence intensity. *n* = 3 animals. All error bars represent standard deviation from the mean.

should be able to release the drug in a controlled fashion, increase drug residence time on the ocular surface, improve the bioavailability of the drug, and improve the local tolerability of the drug. Also, the issue of patient compliance must be seriously considered in ocular drug delivery. For example, if a drug must be given 4−8 times a day for a week when treating a chronic disease, it is very unlikely to be given in a timely fashion. Therefore, development of a controlled release ocular drug delivery system that can release the drug in therapeutically effective concentrations for a longer duration of time (from a day to a week) is highly desired.

This work presents a nanowafer drug delivery platform involving the seamless integration of nanofabrication and drug delivery technologies. A systematic screening of a series of polymers confirmed that PVA is a nonimmunostimulatory polymer that can be used in the fabrication of ocular drug delivery nanowafers, wherein PVA and the drug axitinib work synergistically. The laser scanning confocal imaging and RT-PCR studies have quantitatively demonstrated the enhanced therapeutic efficacy of the nanowafer in terms of inhibiting neovascularization and improved suppression of proinflammatory and proangiogenic factors in an ocular burn induced mouse model. The nanowafer has demonstrated an enhanced efficacy compared to topically applied eye drop treatment. This study also demonstrated that the enhanced therapeutic efficacy is due to the increased drug residence time on the ocular surface, which subsequently diffused into the ocular tissue for therapeutic action. The additive effects of negligible inflammatory potential, mucoadhesivity, controlled drug release, and the therapeutic potential of the drug support the development of a broadly applicable synergistic nanowafer drug delivery system. Furthermore, the nanowafer, at the end of the stipulated drug release time, will dissolve and fade away. The simplicity and efficiency of the nanowafer drug

delivery system provides a new and novel modality for the noninvasive drug administration to the ocular surface. The nanowafer drug delivery system can deliver a wide range of drugs regardless of molecular weight or chemical properties. Development of a nanowafer drug delivery system that can be readily instilled on the ocular surface by the patient's fingertip without any clinical procedure will be not only very convenient but also most desirable for treating eye injuries, infections, chronic dry eye, glaucoma, and other ocular inflammatory conditions. Since, the polymers and drugs used in the development of the nanowafer are already in clinical use, it can be rapidly translated to the clinic for human trials.

MATERIALS AND METHODS

All animals were treated in accordance with the Association of Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Nanowafer Fabrication. Silicon wafers having wells of 500 nm diameter and 500 nm depth were fabricated by e-beam lithography and dry reactive ion etching following a previously published procedure.[23,24] By using the silicon wafer as a master template, poly(dimethylsiloxane) imprints were fabricated. A clear PVA solution (5% w/v) was prepared by dissolving PVA (5 g) in 100 mL of a water/ethanol mixture (4:6) on a stirring hot plate at 70 °C (RT Elite stirring hot plate, Fisher Scientific) until a clear homogeneous solution was formed. Thus, the prepared PVA solution (5 mL) was transferred with a pipet onto a 3 in. diameter PDMS template containing vertical posts and kept in an oven (Isotemp 500 Series Economy Lab Ovens, Fisher Scientific) at 60 °C for 30 min. This procedure resulted in the formation of PVA nanowafers having 500 nm wells. The 500 nm wells in the PVA nanowafer were filled with Axitinib/PVA solution. The Axitinib/PVA solution was prepared by dissolving Axitinib (100 mg in 250 μL of DMSO) in 750 μL of 5% PVA solution. Thus, the prepared Axitinib/PVA solution (200 μL) was transferred onto a PVA nanowafer (3 in. diameter), swiped with a razor blade to fill the wells, and left at room temperature to evaporate the solvent.[27] The drug-filled hydrogel templates were cut into 2 mm diameter discs with a paper punch. The drug content in 2 mm nanowafers was evaluated by HPLC. The nanowafers thus prepared were used for *in vivo* experiments in mice.

Doxy Nanowafer Instillation, Imaging, and Tear Collection. A nanowafer was placed on top of each cornea with a forceps while observing under a stereomicroscope followed by wetting with 5 μL of balanced salt solution (BSS). The mice were anesthetized by ketamine (100 mg/kg) and xylazine (10 mg/kg) injection. The nanowafers dissolved in approximately half an hour. Since doxycycline is a fluorescent drug, Doxy nanowafers were monitored hourly for 5 h *via* imaging in a stereoscopic zoom microscope (model SMZ 1500; Nikon, Melville, NY, USA), with a fluorescence excitation at 470 nm.

Tear fluid was collected at regular intervals by instilling 2 μL of BSS on the ocular surface. After a few seconds the tears were collected from the conjunctival sac close to the lacrimal punctum.[35] The tear fluid and isotonic solution were collected with a 1 μL volume glass capillary tube (Drummond Scientific, Broomhall, PA, USA) by capillary action from the interior tear meniscus in the lateral canthus. The tear washings from a group of 15 mice were pooled, centrifuged, and stored at −80 °C prior to HPLC analysis to determine the drug concentration.

Intravital Imaging of Doxy Nanowafers. Mice instilled with doxycycline nanowafers after 24 h were anesthetized with ketamine and xyalizine and imaged under a Nikon ECLIPSE intravital microscope (Nikon, Melville, NY, USA). Images were captured with a resolution of 1024 × 768 pixels with X10 Nikon objectives.

HPLC Analysis. HPLC experiments were performed on a Shimadzu Prominence HPLC system. The analytical column was a Kinetex 5uXB-C18 100A (150 mm × 4.6 mm) from Phenomenex. The system was equipped with autosampler, in-line degasser, and column oven set at room temperature. The mobile phase for doxycycline analysis was a mixture of 5% acetic acid (55%) and methanol (45%) (Sigma-Aldrich, St. Louis, MO, USA). Injection volume was 5 μL, the flow rate was 1.0 mL/min, and the pressure was lower than 2500 psi.

Determination of total drug content in a nanowafer: The total amount of a drug loaded in the nanowafer was determined by dissolving an accurately weighed nanowafer in 1 mL of PBS solution, followed by addition of 2 mL of ethanol. The precipitated polymer was removed by centrifugation. The clear solution was filtered through a 0.2 μm syringe filter, analyzed by UV-HPLC, and compared with the standard curve to quantify the total drug content in the nanowafer. This experiment was performed in triplicates.

Study of drug release kinetics of the nanowafers by HPLC analysis: Each sample was filtered through a 0.2 μm syringe filter and subjected to HPLC analysis. The UV detection wavelength for doxycycline was 274 nm. The drug concentration was calculated by comparing the peak area of standards and sample.

Ocular Burn Mouse Model and Nanowafer Treatment. Naive female C57BL/c mice 6 to 8 weeks of age (The Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized with an intraperitoneal injection of the rodent combination anesthesia previously mentioned, combined with topical anesthesia of the right eyes by 0.5% proparacaine. Whatman filter paper (2.5 mm diameter) was briefly soaked in 1 N NaOH solution and then placed on the right corneas for 30 s and then rinsed with 20 mL of BSS. Mice corneas were monitored daily using a slit lamp microscope (model 30-99-49, Zeiss, Oberkochen, Germany) for 14 days, and images were recorded by an attached Nikon D40X digital camera. For treatment, each day a specific nanowafer was placed on top of the ocular burn cornea of an anesthetized mouse corresponding to the treatment group. All mice then received 5 μL of BSS on the ocular burn cornea, including control groups.

Corneal Fluorescein Staining. The extent of corneal wound closure was examined by corneal fluorescein staining. On days 1, 3, 5, 7, 9, and 11 after ocular burn, mice were anesthetized with an intraperitoneal injection of the above-mentioned rodent combination anesthesia. A 1 μL amount of fluorescein (0.1%) was instilled on the ocular burn corneas for 1 min, followed by rinsing with 1 mL of BSS. Images were recorded by a stereoscopic zoom microscope (model SMZ 1500; Nikon), with a fluorescence excitation at 470 nm.

Corneal Whole Mount Staining. Fourteen days after the ocular burn, eyes were enucleated for corneal whole mount staining with some modifications.[42] Briefly, corneas including limbal area were dissected from freshly enucleated eyes, and surrounding conjunctiva, Tenon capsule, uvea, and lens were carefully removed, followed by making four slits with a scalpel blade at 90°, 180°, 270°, and 360° to flatten out the corneas, then fixed in 4% (wt/vol) paraformaldehyde solution at room temperature for 1 h. Tissues were blocked with 10% goat serum and 0.5% Triton X-100 prepared in PBS for 1 h. Rat anti-mouse CD31 antibody (1:300) (BD Biosciences, San Jose, CA, USA) supplemented with 5% goat serum and 0.1% Triton X-100 was added to the tissues and allowed to incubate at 4 °C for 3 days. After a series of washing with PBS and blocking with the above-mentioned solution, the tissues were incubated with Alexa-Fluor 594-conjugated goat anti-rat secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) in a dark chamber for 1 h at room temperature. The tissues were then mounted on slides using Fluoromount G (Southern Biotech, Birmingham, AL, USA) containing DAPI (1:300) (Life Technologies, Grand Island, NY, USA).

Laser Confocal Fluorescence Imaging and 3D Representations of Whole-Mounted Corneas. Images of whole-mounted corneas were obtained by stitching individual Z-stack images (~11 × 11) acquired in a Nikon Eclipse Ni confocal microscope provided with a 20× objective (Plan APO20X-0.75/OFN25-DIC-N2 by Nikon) and a 561 nm laser (blood vessel detection, red). Each Z-stack was captured using nonresonant galvano scanners, 512 × 512 pixel size, unidirectional scan, 0.5 scan speed, 2.2 pixel dwell, 0.9 μm Z-space, and 19.2 μm pinhole size. Images were stitched by NIS Elements software, and some of them were deconvolved in the NIS Deconvolution module in order to improve the signal. Images were further processed with IMARIS 7.7.2 (Bitplane AG, Zurich, Switzerland) software for 3D representations and volume calculations. Confocal images were masked using the surpass mode, and the surface function set it up with 3.0 μm surface grain size and 10 μm for the diameter of the largest sphere parameters. The thresholds were adjusted manually for each image. Blood vessel volumes of the 3D representations were calculated using the Statistic function. Data in figures are shown as mean ± SEM of each treatment repeated in triplicates. Statistical significance of comparison of mean values was assessed by one-way-ANOVA followed by Tukey's test for multiple comparisons. Mean differences of the groups were considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Quantitative Reverse Transcription–Polymerase Chain Reaction. Mice were sacrificed 5 days after OB with different treatment. After enucleating, corneas were excised and dissected from surrounding conjunctiva and uvea. Pools of five corneas were prepared in triplicate for each treating group. RNA was extracted by a previously reported procedure with RNeasy MicroKit columns (Qiagen, Valencia, CA, USA).[43] Samples were treated with DNase (Qiagen) and stored at −80 °C. The first-strand cDNA was synthesized from 1.0 μg of RNA with Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Princeton, NJ, USA) and random hexamers (Applied Biosystems, Foster City, CA, USA). RT-PCR was performed using TaqMan Gene Expression Master Mix and Assays (Applied Biosystems). Specific primers from Applied Biosystems were used to quantify gene expression levels. The threshold cycle for each target mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA and averaged. Three groups of five-cornea pools were processed for each group.

Conflict of Interest: The authors declare no competing financial interest.

Supporting Information Available: Slit-lamp images of the ocular burn induced corneas demonstrating the therapeutic effect of the drug nanowafers on corneal neovascularization; drug escalation study to determine the maximum tolerated drug dose in the nanowafer; and the PCR data demonstrating the nonimmunogenic nature of the Axi nanowafer. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Appendix 2: Journal publication

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Dexamethasone nanowafer as an effective therapy for dry eye disease

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ABSTRACT

Dry eye disease is a major public health problem that affects millions of people worldwide. It is presently treated with artificial tear and anti-inflammatory eye drops that are generally administered several times a day and may have limited therapeutic efficacy. To improve convenience and efficacy, a dexamethasone (Dex) loaded nanowafer (Dex-NW) has been developed that can release the drug on the ocular surface for a longer duration of time than drops, during which it slowly dissolves. The Dex-NW was fabricated using carboxymethyl cellulose polymer and contains arrays of 500 nm square drug reservoirs filled with Dex. The in vivo efficacy of the Dex-NW was evaluated using an experimental mouse dry eye model. These studies demonstrated that once a day Dex-NW treatment on alternate days during a five-day treatment period was able to restore a healthy ocular surface and corneal barrier function with comparable efficacy to twice a day topically applied dexamethasone eye drop treatment. The Dex-NW was also very effective in down regulating expression of inflammatory cytokines (TNF-α, and IFN-γ), chemokines (CXCL-10 and CCL-5), and MMP-3, that are stimulated by dry eye. Despite less frequent dosing, the Dex-NW has comparable therapeutic efficacy to topically applied Dex eye drops in experimental mouse dry eye model, and these results provide a strong rationale for translation to human clinical trials for dry eye.

1. Introduction

Dry eye is one of the most common eye diseases that affects approximately 30–40 million people in the United States alone and represents a major public health problem [1,2]. It is accompanied by eye irritation, blurred vision, light sensitivity, and ocular surface epithelial disease [3,4]. Also, it is one of the primary causes for clinical visits and approximately 30% of patients report symptoms of mild or chronic dry eye disease [5–7]. The causative factors include inflammation, hormonal imbalance, and aging. In addition, dry eye is a common complication of LASIK surgery [8,9]. A steady growth in computer screen-related visually demanding tasks and contact lens use can exacerbate dry eye [3,10]. Many of these causative factors adversely impact one or more components of the lacrimal functional unit including the ocular surface, lacrimal glands, meibomian glands, and the interconnecting neural network [11,12]. Mild dry eye is primarily treated with artificial tear drops that hydrate and lubricate the eye, do not have any pharmacologic activity, and provide only temporary relief [13,14]. Chronic dry eye is associated with inflammation that often responds to topically applied corticosteroids and cyclosporine-A eye drops [15–17]. Because of the rapid clearance, eye drops must be administered several times in a day resulting in a high-and-low drug concentration profile and potentially toxic side effects [18,19]. Also, frequent dosing is inconvenient and can cause discomfort that can compromise compliance. To address these issues, several new strategies have been developed [20]. Recently, nanoparticle formulations have been used in ocular drug delivery [21–24]. The rapid clearance of these formulations from the eye contributed to their limited drug efficacy. As another advancement, drug soaked contact lenses were developed [25–27]. These contact lenses released the drug very rapidly. Despite these methodological advances, controlled delivery of drugs in therapeutically effective concentration to tissues in the anterior segment of the eye, such as cornea and conjunctiva, is still very inefficient. These limitations have lead to a call for the development of a novel nanowafer therapeutic with extended release attributes and enhanced efficacy to treat dry eye disease.

The nanowafer is a tiny disc-like or rectangular membrane that contains drug loaded nano-reservoirs (Fig. 1). The nanowafer can be readily applied on the eye by the patient's finger tip without any clinical procedure (Fig. 1). Although, instillation of drug solution or nanoparticle suspension as topical eye drop formulation is the simplest mode of ocular drug delivery, application of a nanowafer on the eye is as easy as applying a contact lens. The nanowafer, after its instillation on the ocular surface, can release the drug for a longer duration of time than eye drops, thus improving its therapeutic efficacy. During the course of the drug release, the nanowafer slowly dissolves. In this study, hydrogel-forming carboxymethyl cellulose (CMC) polymer was chosen for the nanowafer fabrication because of its water solubility, mucoadhesiveness, and use as an active ingredient in artificial tear eye drops [28,29]. The mucoadhesive

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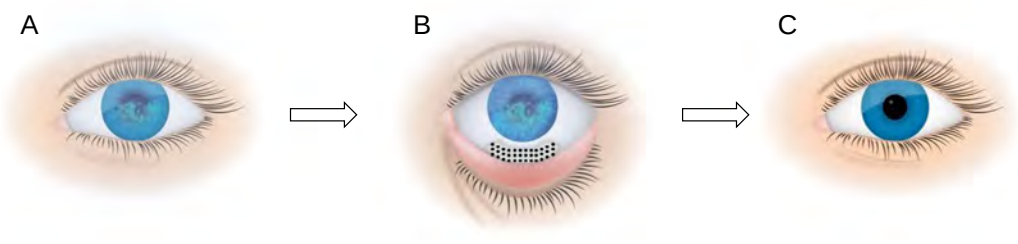

Fig. 1. Schematic of the nanowafer drug delivery to treat dry eye disease. (A) dry eye; (B) instillation of a nanowafer on the conjunctiva; and (C) restoration of a healthy cornea.

property of CMC facilitates quick adhesion and retention of the nanowafer on the ocular surface to withstand reflex tearing and blinking without being displaced. Furthermore, CMC stimulates re-epithelialization of the cornea through its binding to the matrix proteins [30]. The nanowafer contains arrays of nano-reservoirs that are filled with a glucocorticosteroid dexamethasone (Dex). Dex was chosen to fill the nanowafers because of its potent anti-inflammatory properties and has well documented efficacy for treating ocular inflammation [31]. It inhibits the production of inflammatory cytokines, chemokines, and decreases the synthesis of matrix metalloproteinase MMP-3, in addition to other factors [32]. Dex has been successfully used to treat dry eye related corneal epithelial diseases [33].

2. Materials and methods

2.1. Materials

Sodium carboxymethyl cellulose (CMC, MW 90,000) and HPLC solvents (acetonitrile) were obtained from Sigma Aldrich. USP grade dexamethasone sodium phosphate (Dex) was obtained from Spectrum Chemicals. PCR reagents and Oregon Green Dextran (72 KDa) were purchased from Life Technologies.

2.2. Animals

All animals were treated in accordance with the Association of Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Female C57/BL6 mice (6–8 weeks old) were purchased from the Jackson Laboratory.

2.3. Nanowafer fabrication

The nanowafers were fabricated according to a previously published procedure with slight modifications [34–36]. A clear CMC solution (4% w/v, 12 mL) was transferred with a pipette onto a polydimethylsiloxane (PDMS) imprint (3″ diameter) containing square posts (of 500 nm × 500 nm, 500 nm high) placed on a flat glass plate and left to dry at room temperature. Thus formed CMC nanowafer was carefully peeled away from the PDMS imprint. The concentration of the polymer solution can be varied to obtain the required thickness of the nanowafer. The CMC nanowafer obtained was ~3″ diameter, ~75 μm thick, and has arrays of wells (500 nm × 500 nm, 500 nm deep).

The CMC wafers were filled with Dex by transferring a thick solution of dexamethasone-CMC with micropipet onto the nanowafer followed by gently swiping with a Teflon swiper. A similar procedure was used for filling the CMC wafers with fluorescein (a green fluorescent dye). The drug or dye filled wells are open on one side of the nanowafer. The open face of the nanowafer was placed in direct contact with the ocular surface, so that the drug molecules can directly diffuse into the ocular tissue. The drug/dye filled nanowafers were punched into 2 mm diameter discs and used in the in vitro and in vivo experiments. Each 2 mm diameter nanowafer contained 5.027×10^5 wells.

2.4. In vitro drug release study

Accurately weighed Dex-NWs were placed inside dialysis tubes (MWCO 2000). Each loaded dialysis tube was placed inside a 5 mL Eppendorf tube containing phosphate buffered saline solution (PBS, pH 7.4) and constantly shaken at 37 °C. Aliquots were obtained at different time points and analyzed using a Shimadzu Prominence UV–HPLC system with a Kinetex 5uXB-C18 100A (150 mm × 4.6 mm) column from Phenomenex. Fresh PBS was added to replace the aliquot extracted. Each sample was filtered through a 0.2 μm syringe filter, and drug concentration was calculated by comparing the peak area of standards and sample detected at 240 nm. The UV–HPLC system was equipped with autosampler, in line degasser, and column oven set at room temperature. The mobile phase for Dex analysis was a mixture of 0.1 M monosodium phosphate (90%) at pH 4.6 and acetonitrile (10%). Injection volume was 5 μL, the flow rate was 0.8 mL/min, and the pressure was lower than 2500 psi. The total drug content in the nanowafer was determined by dissolving an accurately weighed nanowafer in 1 mL PBS solution and 2 mL of ethanol to precipitate the polymer. The suspension was centrifuged to remove the polymer. The clear solution was filtered through a 0.2 μm syringe filter followed by UV–HPLC analysis. The total drug content in the nanowafer was quantified by comparing with the standard curve. This experiment was performed in triplicates.

2.5. Dex-NW instillation and tear collection

Female C57/BL6 mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The mice were treated with either one Dex-NW containing 10 μg of Dex placed on inferior bulbar conjunctiva or 2 μL eye drops (containing 10 μg of Dex in 2 μL of 0.1% CMC solution). Tear fluid was collected at regular intervals. 2 μL of balanced salt solution (BSS) was instilled on the ocular surface, after a few seconds, the tears, mixed with the isotonic solution, were collected from the conjunctival sac close to the lateral canthus with a 1 μL volume glass capillary tube (Drummond Scientific). The tear washings from a group of 15 mice were pooled, centrifuged, and stored at −80 °C prior to drug quantification. UV–HPLC analysis was performed following the same parameters established for the in vitro experiments except, using 10 μL as the injection volume.

2.6. Experimental mouse dry eye model

Desiccating stress (DS) was used to induce experimental dry eye in female C57/BL6 mice, six to eight weeks of age, by subcutaneous injection of 0.5 mg/0.2 mL scopolamine hydrobromide (Sigma Aldrich) into alternating hindquarters administered four times a day (8:30 a.m., 11 a.m., 1 p.m. and 4:30 p.m.) to inhibit tear secretion, exposure to an air draft, and <30% ambient humidity. Mice were euthanized after five days of desiccating stress (DS) treatment [37]. A group of age- and gender-matched mice that were housed in normal environmental conditions were used as non-stressed (NS) controls.

2.7. Evaluation of corneal smoothness

Reflected images of a white ring from the fiber-optic ring illuminator of the stereoscopic zoom microscope (SMZ 1500; Nikon) were obtained immediately after euthanasia. This ring light is firmly attached and surrounds the bottom of the microscope objective. Because the illumination path is nearly coincident with the optical axis of the microscope, the viewing area is evenly illuminated and nearly shadowless. The projected ring light will reflect off a wet surface and the regularity of the reflected ring depends on the smoothness of the ocular surface [38–40].

2.8. Corneal barrier function measurement

On the morning of the fifth day of DS, corneal staining was measured by Oregon Green Dextran (OGD). 0.5 μL of OGD was instilled on the cornea of both eyes and the mouse was left in the dark for 1 min before euthanization. The eyes were then washed with 2 mL of BSS. Excess liquid was blotted off the eye with tissue paper and digital images were captured and the mean fluorescence intensity within a 2 mm central corneal ring was measured with NIS Elements (Nikon). The data are presented as mean ± SEM of fluorescence gray levels from three independent experiments using 5 mice per group per experiment [38].

2.9. RNA isolation and real time RT-PCR

The corneal epithelium was scraped off with a scalpel and the tissue was collected for PCR analysis. Total RNA from corneal epithelium was isolated using a QIAGEN RNeasy Plus Micro RNA isolation kit (Qiagen) following the manufacturer's protocol. After isolation, the concentration of RNA was measured and cDNA was synthesized using the Ready-To-Go™ You-Prime First-Strand kit (GE Healthcare). Real time PCR was performed using specific Taqman probes for MMP-3 ((*Mmp3*) (Mm00440295_m1)), CCL-5 ((Ccl5) (Mm00445235-m1)), CXCL-10 (Cxcl10) (Mm01302427_m1), TNF-α ((*Tnfa*) (OriGene MP217748)), and IFN-γ ((*Ifng*) (Mm00801778_m1)) genes (Taqman Universal PCR Master Mix AmpErase UNG) in a commercial thermocycling system (StepOnePlus™ Real-Time PCR System, Applied Biosystems) according to the manufacturer's recommendations. The results were analyzed by the comparative threshold cycle method and normalized by beta 2 microglobulin (B2M) as the control.

2.10. Statistical analysis

Prism 6.0 software (GraphPad Software Inc.) was used for statistical analysis. One-way analysis of variance (ANOVA) was used to determine overall differences among groups, followed by a post-hoc test (Tukey's post hoc). An unpaired *t*-test is used to evaluate statistical differences between 2 experimental groups. The statistical significance was considered to be $P \leq 0.05$ and data are presented as mean ± SEM.

3. Results and discussion

In this article, we describe the development of a nanowafer therapeutic and demonstrate its in vivo efficacy in an experimental mouse dry eye model (dry eye mice) [37,38]. The nanowafers were fabricated

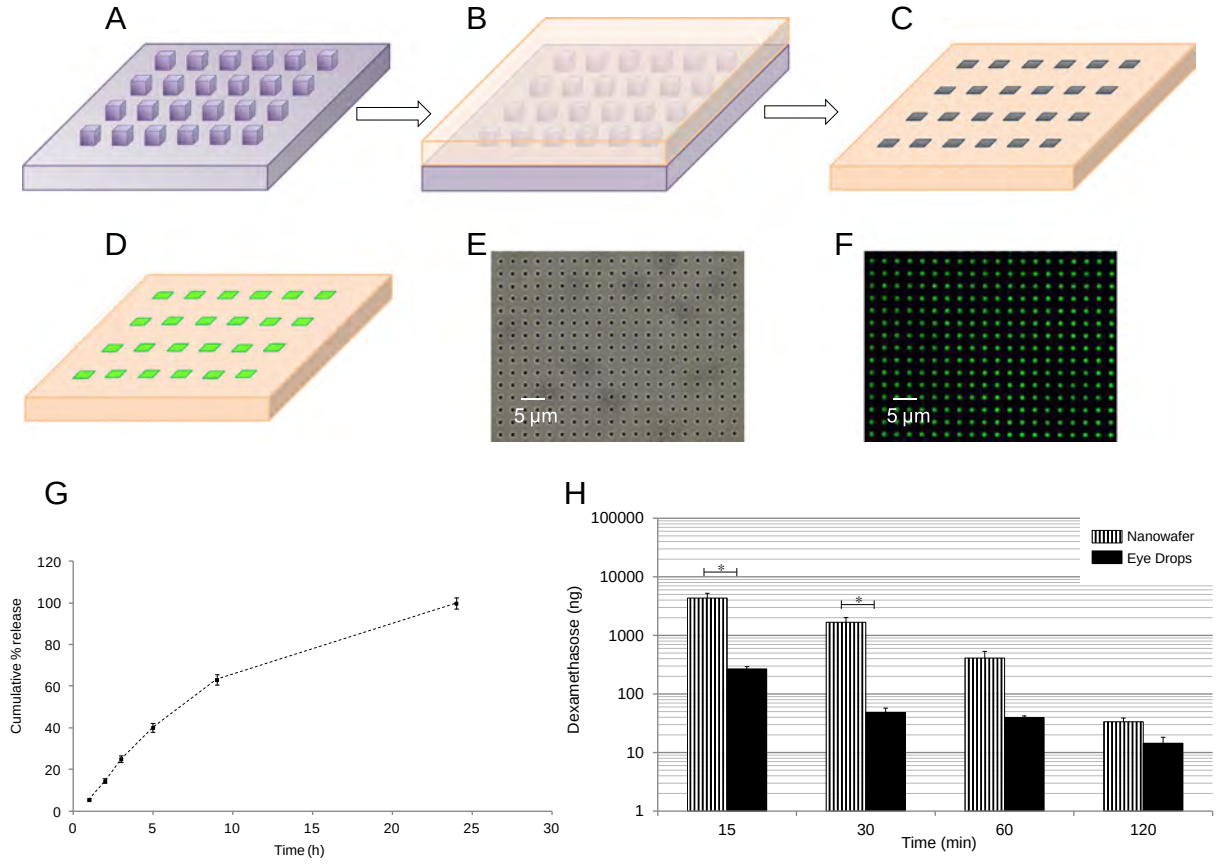

Fig. 2. Nanowafer fabrication and the drug release from Dex nanowafer. Fabrication of nanowafer by hydrogel template strategy: (A) a PDMS template containing vertical posts; (B) preparation of a CMC imprint of the PDMS template; (C) a CMC nanowafer; (D) a CMC nanowafer filled with drug/dye; (E) bright field image of a CMC nanowafer; (F) fluorescence image of a CMC nanowafer filled with fluorescein. Dex release from a nanowafer: (G) In vitro drug release profile; and (H) Dex concentration measured in tear samples collected from eyes treated with Dex-NW or Dex eye drops. *$P < 0.05$.

by hydrogel template strategy [34–36]. A schematic of the nanowafer fabrication procedure has been presented in Fig. 2A–F. Carboxymethyl cellulose (CMC) was chosen for the nanowafer fabrication because of its water solubility, mucoadhesiveness, and use as a constituent in artificial tear eye drops [28–30]. The nanowafer contains arrays of wells that were filled with a glucocorticosteroid dexamethasone (Dex, MW = 392 g/mol). For conducting in vivo studies in dry eye mice, 2 mm diameter nanowafers were fabricated to be placed on the conjunctiva.

The Dex loaded nanowafers (Dex-NW) were examined for their in vitro drug release kinetics by HPLC. The total Dex content in the Dex-NW was 10 μg. The initial release after 5 h was ~40% and the drug release continued for 24 h (Fig. 2G). To assess the drug release from the Dex-NW after its instillation on the conjunctiva, tear samples were collected at hourly intervals and analyzed for Dex concentration by HPLC. This study revealed the presence of Dex in tear samples for up to 2 h at significantly greater concentrations than eyes treated with Dex eye drops containing the same amount (10 μg in 2 μl) of the drug (Fig. 2H). Also, in the case of Dex release from the nanowafer, more drug was present in the first hour tear samples compared to the second hour samples. Because of the ocular surface barriers, such as reflex tearing and tight epithelial junctions, the drug diffusion into the cornea is very slow in the beginning, hence more drug was present in the first hour tear samples. However, with longer drug residence time provided by the nanowafer, more drug penetrates into the cornea. This results in a decrease in drug concentration in the tear samples collected at later time points.

To study the efficacy of the nanowafer in improving the drug molecular residence time on the ocular surface and its subsequent diffusion into it, fluorescein (a green fluorescent dye, MW = 332 g/mol) loaded nanowafers (Flo-NW) were fabricated. The Flo-NWs were instilled on the corneas of healthy mice and were examined by fluorescence imaging at every hour for 5 h. The ocular surface was initially non-fluorescent (Fig. 3A). After the placement of a nanowafer, the fluorescein molecules were slowly released on the cornea and green fluorescence was observed. Since, the fluorescein released from the nanowafer is in direct contact with the cornea, most of the drug will be able to penetrate into it. After 4 h, the green fluorescence of the cornea started to fade because of the diffusion of fluorescein molecules into the anterior chamber and its clearance by tear secretion (Fig. 3B–G). Once the fluorescein molecules pass through the cornea and reach the aqueous humor in the anterior chamber, they are cleared through the trabecular meshwork. In comparison, eyes treated with fluorescein eye drops were non-fluorescent after 5 min, indicating its rapid clearance from the ocular surface (Fig. 3H–L). Also, after instillation of the fluorescein drops on the eye, within a few minutes, most of it is concentrated on the eye lids, indicating its clearance from the ocular surface, compared to the nanowafer drug release, wherein only a small amount of fluorescein concentrated around the eyelids and most of it in the eye. This study has qualitatively demonstrated the ability of nanowafer to release the drug for a few hours thus improving the drug residence time on the cornea.

The in vivo efficacy of Dex-NW was evaluated in dry eye mice. A Dex-NW was placed on the inferior bulbar conjunctiva of a mouse (on days 1 and 3) that was subjected to desiccating stress for five days with no topical treatment. The efficacy of the Dex-NW was evaluated by corneal smoothness, corneal barrier function, and expression of pro-inflammatory cytokines, chemokines, and MMPs and compared with control CMC-NW (without Dex) treated and untreated dry eye mouse groups.

Chronic dry eye disease can desiccate the ocular surface and create corneal epithelial erosions that alter corneal smoothness and

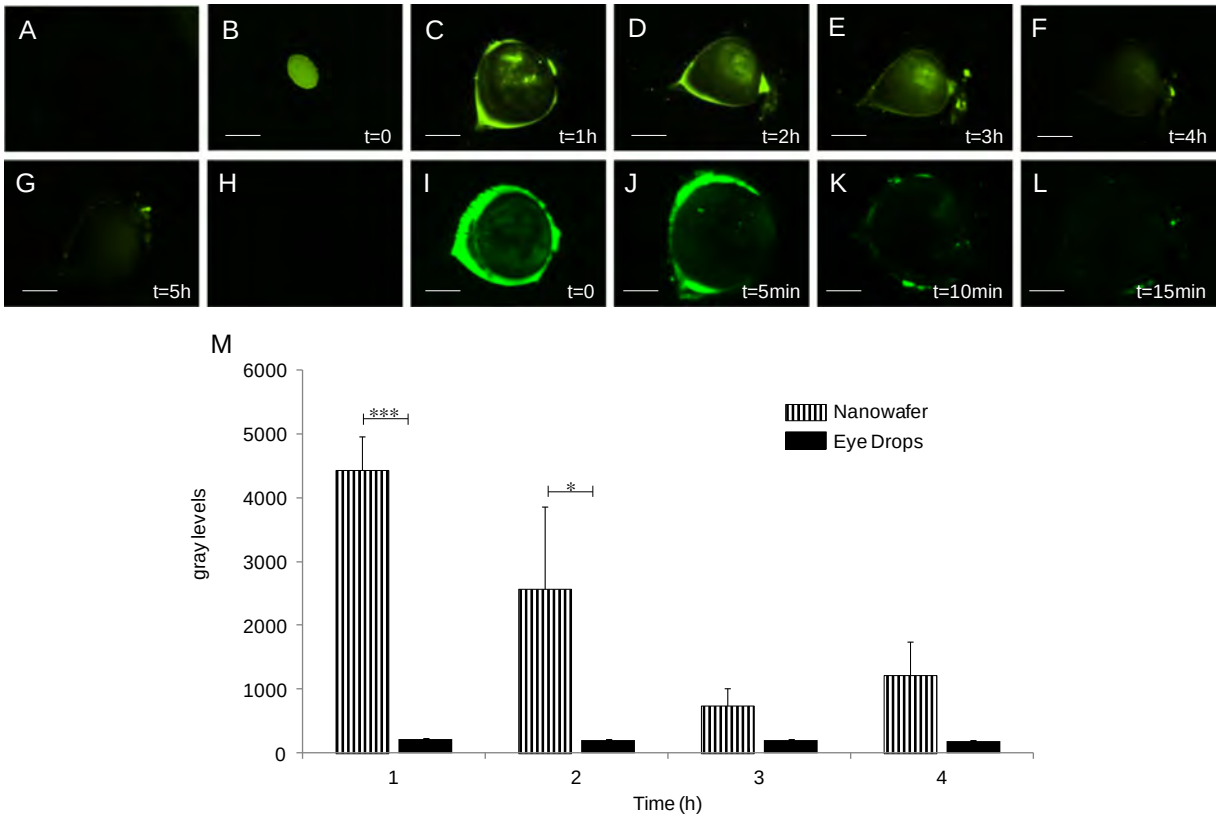

Fig. 3. Nanowafer improves the drug residence time on the cornea. (A) Fluorescence micrograph of the eye prior to fluorescein nanowafer (Flo-NW) instillation; (B–G) fluorescence micrographs depicting the presence of fluorescein dye at the specified time points in the corneal tissue after Flo-NW instillation; (H) fluorescence micrograph of the eye prior to fluorescein eye drops instillation; (I–L) rapid clearance of fluorescein eye drops within 5 min. (M) A plot depicting the fluorescence intensities (n = 3).*P < 0.05, ***P < 0.001. All error bars represent standard error of the mean. Scale bar: 1 mm.

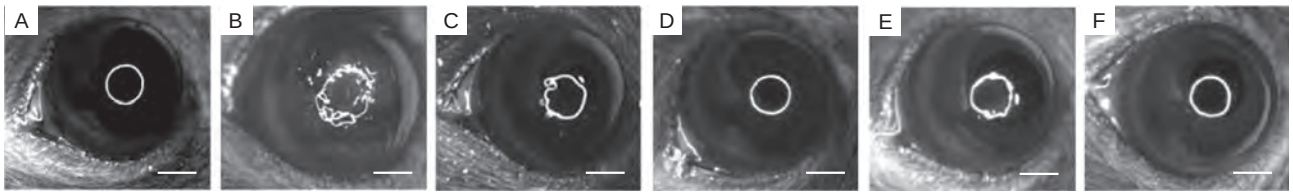

Fig. 4. Maintenance of a smooth corneal surface by Dex-NW treatment observed by the reflection of a ring of light in mice. (A) Healthy eye; (B) Desiccating stress induced dry eye; (C) CMC-eye drops; (D) Dex-eye drops; (E) CMC-NW; and (F) Dex-NW. Scale bar: 1 mm.

permeability [38–40]. Accordingly, corneal smoothness was monitored as a study parameter for the evaluation of nanowafer efficacy. The regularity of a white-light ring reflecting off the mouse cornea was used to evaluate corneal smoothness. A regular circular ring is reflected off the smooth normal corneal surface. In dry eye mice treated with control CMC nanowafers, the reflected rings were very irregular, while regular and uniform ring reflections were observed from eyes treated with Dex-NW, indicating maintenance of a smooth normal corneal surface (Fig. 4). Improved corneal smoothness over control affirms the efficacy of Dex-NW. These results indicate that the drug release from the nanowafer is effective in restoring or maintaining a healthy corneal surface that is subjected to desiccation.

To study the therapeutic effect of Dex-NW on maintaining a healthy corneal surface in mice exposed to DS, corneal epithelial barrier function was measured by staining the cornea with green fluorescent Oregon Green Dextran (OGD, 70 kDa). Corneal epithelial disruptions created due to the damage or loss of epithelial cells was visualized with the dye, where fluorescence intensity positively correlates with the degree of disruption in corneal barrier function [38]. For the corneal epithelial barrier function measurement, OGD was delivered as topical eye drops. Because the mice are exposed to DS, the corneas develop epithelial disruptions due to the damage or loss of epithelial cells. The corneal surface is no longer smooth with tight epithelial junctions. Upon instillation of OGD eye drops on these corneas, the OGD molecules will rapidly (<1 min) penetrate into the corneal tissue through the epithelial disruptions compared to healthy and drug treated corneas. Because of the short duration of the eye drops on the cornea (1 min) and the low blink rate of mouse, most of the eye drop stays on the ocular surface and does not get washed away.

Compared to a healthy eye, the dry eye mice absorbed more OGD. This study revealed that the corneal uptake of OGD significantly increased after five days in dry eye mice (Fig. 5). In contrast, treatment with Dex-NW or Dex drops maintained corneal epithelial barrier function. Treatment of dry eye mice with Dex eye drops (0.1%, 2 μL) administered twice a day for five days or instillation of Dex-NW on the first and third days during five-days of experimental dry eye maintained OGD staining at baseline levels. No difference in efficacy was observed between the Dex-NW and Dex drops treated groups, despite the lower dosing frequency for the Dex-NW. These results confirm the ability of the nanowafer to deliver Dex in therapeutic concentrations to the ocular tissue. Interestingly, CMC eye drops appear to be slightly more effective, although not significant, compared to the CMC nanowafer in maintaining the corneal barrier function (Fig. 5G). This could be because of the ability of the CMC polymer molecules in eye drop formulation delivered twice daily to quickly bind to the corneal epithelium thus filling some of the corneal epithelial disruptions caused by the desiccating stress, compared to the CMC nanowafer which was applied only twice during the 5 day treatment period [30]. Also, when an eye drop is instilled on the ocular surface, it will have some wetting/moisturizing effect on the surface regardless of whether it has any effective ingredients (vehicle effect). The nanowafer on the other hand did not produce as much vehicle effect as it was instilled only twice during the treatment period (on days 1 and 3).

Dry eye is a multifactorial disease. It activates an innate immune response in the cornea and conjunctiva that ultimately affects the corneal epithelium by producing chemokines and cytokines. Chemokines CXCL-9, -10, -11 attract T helper cells (type 1) which produce IFN-γ, while other chemokines, such as CCL5 attract innate immune cells [41]. Important immune mediators in the conjunctiva also include cytokines IFN-γ and TNF-α [42,43]. In particular, IFN-γ is known to cause conjunctival goblet cell loss and apoptosis of the ocular surface epithelium [44–47]. The gene expression of these molecules was evaluated due to their importance in the pathogenesis of dry eye. Matrix metalloproteinase MMP-9 and its physiological activator MMP-3 have been found to increase in the ocular surface epithelium of both human and experimental dry eye [48–51]. The MMPs break down a variety of substrates, including components of the corneal epithelial basement membrane and tight junction proteins that maintain corneal epithelial barrier function. MMPs have also been detected in the tear fluid of both dry eye patients and dry eye mice [39].

Inhibition of inflammatory cytokine and MMP expression confirms the efficacy of Dex-NW. Instillation of Dex-NW on the first and third days effectively inhibited expression of inflammatory cytokine and MMP production in the corneal epithelium compared to CMC-NW (control). The slight anti-inflammatory effect of CMC nanowafer is possibly due to the ability of CMC polymer molecules to bind to the corneal epithelium and protect it against the pro-inflammatory effects of the DS and modulate the healing process [30]. The efficacy of the Dex-NW (administered on first and the third days) was equal to twice a day topical administration of Dex eye drops for the five-day treatment period

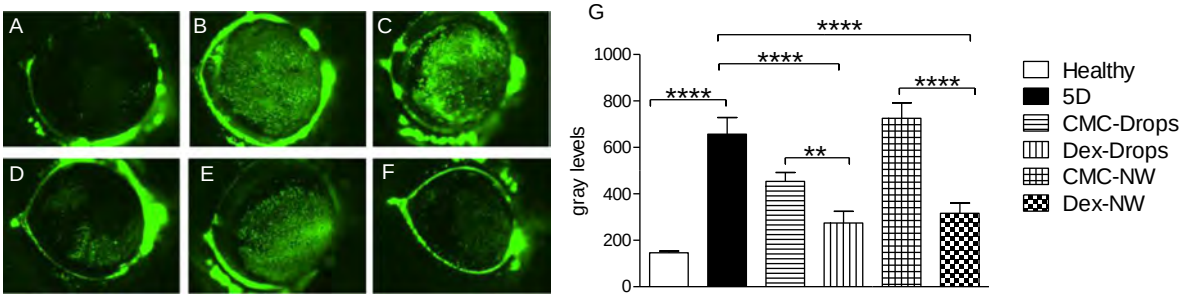

Fig. 5. Measurement of corneal epithelial barrier function. (A) Healthy eye; (B) subjected to five days of desiccating stress (5D); (C) CMC drops; (D) Dex drops; (E) CMC-NW; (F) Dex-NW; and (G) a plot depicting the fluorescence intensities.**P < 0.05, ****P < 0.0001. All error bars represent standard error of the mean.

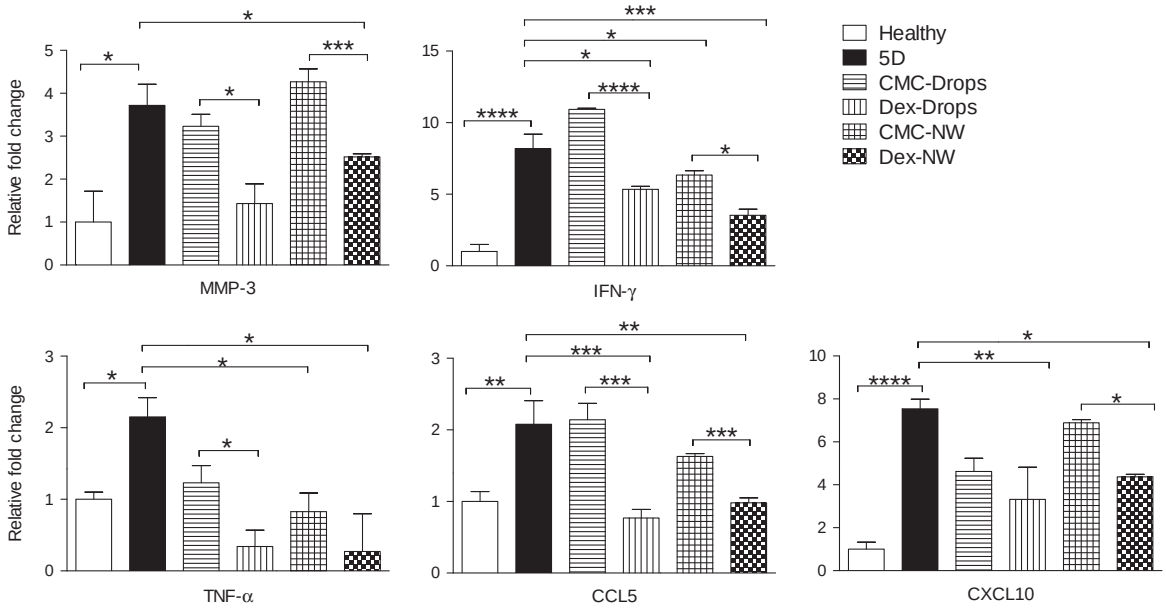

Fig. 6. RT-PCR analysis revealing down regulation of drug target genes by Dex-NW in the corneal epithelium after 5 days of desiccating stress is comparable to that of twice a day topical Dex eye drop treatment. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. All error bars represent standard error of the mean.

(Fig. 6). Both Dex-NW and Dex eye drop treatment inhibited the production of MMP-3, CCL-5, CXCL-10, TNF-α, and IFN-γ. Overall, the Dex-NW was effective in inhibiting inflammatory cytokine expression at the same level achieved with twice a day topically administered Dex eye drops.

4. Conclusions

Despite advances in the therapy of dry eye disease over the past two decades, challenges still remain in delivering therapeutic levels of anti-inflammatory drugs at a convenient dosing schedule and optimum tissue concentrations. This study evaluated the nanowafer drug delivery system to deliver Dex to the ocular surface. The Dex-NW was able to enhance diffusion of a corticosteroid into the cornea and maintain a smooth, healthy corneal surface with intact barrier function in mice with experimentally induced dry eye. In addition, the Dex-NW was effective in suppressing expression of inflammatory mediators associated with dryness of the cornea. The Dex-NW treatment was well tolerated on the mouse eye. Administration of Dex-NW only twice during the five-day treatment had equivalent efficacy to twice a day topical administration of Dex eye drops during same treatment period. The less frequent dosing schedule of the Dex-NW will improve convenience and enhance treatment compliance among dry eye patients. Furthermore, release of dexamethasone from the nanowafer can be adjusted to the minimal effective concentration to minimize the drug related toxicity of corticosteroids that can cause glaucoma and cataract at higher concentration. These results confirm the therapeutic efficacy and translational potential of the nanowafer drug delivery system to treat dry eye disease. Upon further development, Dex-NW can provide a simple and effective treatment with a more convenient dosing schedule than eye drops for dry eye disease.

Acknowledgments

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Appendix 3: Conference paper

Acharya et al, Nanowafer Drug Delivery to Treat Corneal Neovascularization. *Invest. Ophthalmol. Vis. Sci.* 2015; 56(7):5032

studies showed increased internalization of targeted micelles as compared to non-taregeted micelles.
Conclusions: Folic acid conjugated Vitmain E TPGS was successfully synthesized. Highly hydrophobic drugs like FA can be formulated into clear aqueous eye drops. Folic acid targeted micelles can be utilized to efficiently deliver drugs to retinal and other ocular tissues.
Commercial Relationships: Sujay J. Shah, None; **Sulabh Patel**, None; **Ashaben Patel**, None; **Ashim K. Mitra**, None
Support: NIH Grant 2 R01 EY010659-12A2

Program Number: 5031 **Poster Board Number:** C0008
Presentation Time: 3:45 PM–5:30 PM
Functionalized nanoparticle technology for enhanced drug delivery
Jai Parekh, David Freilich, Stephanie Youlios, Sima Parekh, Uday Kompella. EyeTrans Technologies, New York City, NY.
Purpose: Due to short residence time of eye drops, drug bioavailability to the eye surface is low, particularly for poorly soluble and poorly permeable drugs, necessitating frequent dosing in treating dry eye and glaucoma. Following intravitreal injections, target specific delivery is currently not feasible for macromolecule and small molecule drugs useful in treating wet age-related macular degeneration and other back of the eye diseases. To address these unmet needs our objective is to develop novel nanoparticle based technologies for topical and intraocular drug and gene delivery.
Methods: Small nanoparticles technologies capable of drug encapsulation and surface modification with hydrophilic cell recognizing components (functionalized nanoparticles) were designed to enhance mucus penetration as well as epithelial surface recognition and uptake. These technologies licensed by EyeTrans are under development for ocular drug delivery and therapy. The nanoparticle technologies utilize a drug carrier, a ligand on the particle surface to recognize cell surface, and other polymers to enhance delivery or stabilize the particle.
Results: This presentation will describe nanoparticle preparation and evidence to date indicating that surface functionalization enhances ocular surface tissue uptake as well as retinal pigment epithelial cell uptake of nanoparticles in ex vivo/in vitro studies. Further, it will describe evidence indicating that dosing with biodegradable functionalized nanoparticles enhances drug delivery to the tissues of the eye in an animal model.
Conclusions: Nanoparticle and drug delivery can be enhanced by using particle surface features that allow tissue recognition and uptake.
Commercial Relationships: Jai Parekh, EyeTrans Technologies (S); **David Freilich**, EyeTrans Technologies (S); **Stephanie Youlios**, None; **Sima Parekh**, None; **Uday Kompella**, EyeTrans Technologies (P), EyeTrans Technologies (S)

Program Number: 5032 **Poster Board Number:** C0009
Presentation Time: 3:45 PM–5:30 PM
Nanowafer Drug Delivery to Treat Corneal Neovascularization
Ghanashyam Acharya, Xiaoyong Yuan, Daniela Marcano, Crystal Shin, Xia Hua, Lucas Isenhart, Stephen C. Pflugfelder. Ophthalmology, Baylor College of Medicine, Houston, TX.
Purpose: Development of a controlled release nanowafer drug delivery system for treating corneal neovascularization.
Methods: The axitinib-nanowafers (Axi-NW) were fabricated *via* the hydrogel template strategy with a few modifications. The nanowafers thus prepared were tested in ocular burn induced murine model. The corneas were subjected to laser scanning confocal imaging and RT-PCR analysis of gene expression.
Results: The nanowafer is a tiny transparent circular disc containing arrays of drug loaded nanoreservoirs (**Figure 1**). The *in vivo* therapeutic efficacy of the nanowafer was demonstrated by treating corneal neovascularization (CNV) in a murine ocular burn (OB) model. In this study, once a day Axi-5-NW treatment was compared with Axi eye drops (0.1%) administered twice a day for its therapeutic effect in inhibiting CNV in OB mouse model. The Axi-5-NW treatment restricted the proliferation of blood vessels to the limbal area and treated eyes very closely resembled the healthy uninjured cornea. However, the OB controls - PVA-NW and Axi eye drop treated corneas exhibited extensive neovascularization (**Figure 2a-e**). In the case of Axi-5-NW treatment, the amount of drug delivered to the cornea was 5 μg per day, and for axitinib eye drop treatment it was 10 μg per day. Although, eye drop treated mice received twice the drug dosage as those treated with Axi-5-NW, still Axi-5-NW treatment was twice as efficacious as the eye drop treatment (**Figure 2f**). The RT-PCR study revealed that Axi-NW was very effective in downregulating the drug target genes VEGF-A, VEGF-R1, VEGF-R2, PDGFR-A, PDGFR-B, TNF-α, bFGF and TGF-β, compared to the untreated OB and Axi-eye drop treatment.
Conclusions: Once a day axitinib delivery by the nanowafer is more efficacious than the twice a day topical eye drop treatment.

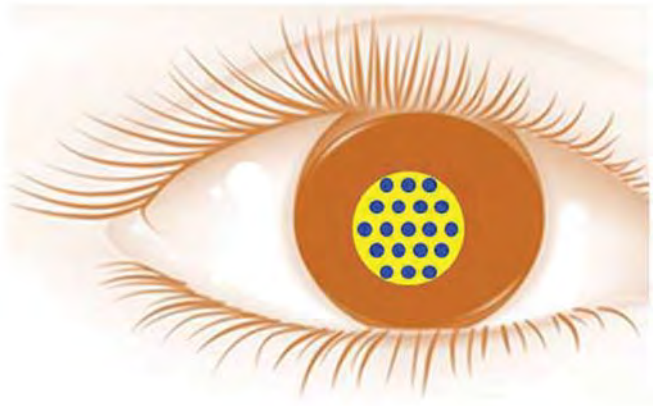

Figure 1. Schematic of the Ocular drug delivery nanowafer instilled on the cornea.

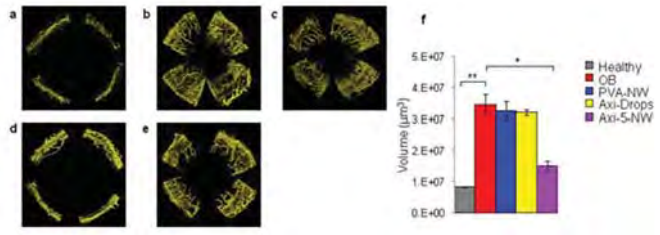

Figure 2. Axitinib-nanowafer is more efficacious than the topical eye drop treatment. Confocal fluorescence images revealing the enhanced therapeutic efficacy of Axi-nanowafer. **a**, Healthy cornea. **b**, OB induced cornea. **c**, PVA-NW. **d**, Axi-NW. **e**, Twice a day Axi-eye drop (0.1%) treatment. **f**, Quantification of corneal neovascularization volume. n = 3 animals,
* P<0.05 vs OB control. All error bars represent standard deviation from the mean.
Commercial Relationships: Ghanashyam Acharya, None; **Xiaoyong Yuan**, None; **Daniela Marcano**, None; **Crystal Shin**, None; **Xia Hua**, None; **Lucas Isenhart**, None; **Stephen C. Pflugfelder**, None

Appendix 4: Conference paper

Shin et al, Nanowafer Drug Delivery for Restoration of Healthy Ocular Surface in Dry Eye Condition. *Invest. Ophthalmol. Vis. Sci.* 2015; 56(7):321.

USA). A control group (4) was left untreated and received no eye drops, but was kept under the same conditions as the therapy groups. Clinical readouts were undertaken weekly (amount of tear fluid; corneal epithelial staining) in combination with a final preparation of conjunctival tissue for counting goblet cell density.
Results: Senescent mice showed a significantly higher increase in epithelial staining after 14 days of EDE and a significantly stronger reduction of tear production compared to young mice. Therapeutic treatment of mice with ScA/F4H5 showed a significantly earlier and stronger increase of tear production and an earlier decrease of epithelial damage following EDE compared to untreated controls, F4H5 alone and Restasis® in both age groups. Clinically senescent mice developed a more severe EDE and efficacy of F4H5/CsA was detected later (after 3 weeks of therapy) than in younger mice (after 1 week).
Conclusions: CsA in F4H5 is highly effective in reducing corneal staining and maintaining conjunctival goblet cells in EDE. Compared to Restasis®, F4H5/CsA was shown to be equally effective, but with a faster therapeutic response in this study. Based on these results first applications in humans are on the way that may lead to a new therapeutic option in treating dry eye disease.
Commercial Relationships: Uta Gehlsen, None; **Tobias Braun**, None; **Claus Cursiefen**, Allergan (C), Gene Signal (C), Novaliq (C); **Philipp Steven**, Novaliq (F)

Program Number: 320 **Poster Board Number:** C0205
Presentation Time: 8:30 AM–10:15 AM
Conjunctival Aquaporins Are Involved in the Resolution of Ocular Phenotype in a Rabbit Dry Eye Model
Dhruva Bhattacharya[1], Yuan Ning[2,3], Fangkun Zhao[2,3], Rongji Chen[1], Jinsong Zhang[2,3], Mingwu Wang[1]. [1]Ophthalmology and Vision Science, University of Arizona College of Medicine, Tucson, AZ; [2]The Fourth Affiliated Hospital of China Medical University, Liaoning Province, China; [3]Eye Hospital of China Medical University, Liaoning Province, China.
Purpose: Longitudinal study of a rabbit dry eye model (over 4-months) found a spontaneous resolution of the dry eye phenotype. Mechanisms of tear fluid compensation in the absence of lacrimal gland were explored.
Methods: A rabbit dry eye model was created by bilateral resection of lacrimal gland (LG), Hardarian gland (HG) and nictitating membrane (NM). Ocular surface of these rabbits (N=8) were characterized by clinical tests (Schirmer tests, fluoroscein test, and rose Bengal staining). In parallel, conjunctival impression cytology (CIC) was done before excision (BE) and every month after excision (AE) over 4-months. Conjunctival molecular biomarkers was studied to supplement the clinical tests, including inflammatory cytokine genes (*interleukin 1*β*: IL-1*β, *tumor necrosis factor: TNF*-α) and *matrix metalloproteinase* (*MMP*-9), using Reverse Transcription-Quantitative-Polymerase Chain Reaction (RT-qPCR). To assess involvement of conjunctiva in restoration of ocular fluid balance, the following genes were assessed by RT-qPCR of CIC: cystic fibrosis trans-membrane conductance regulator: CFTR, sodium potassium chloride co-transporters: NKCC1, sodium potassium ATPase: NKA; epithelial sodium channels: ENaCα and water transporters: Aquaporins (AQP4, AQP5).
Results: Dry eye phenotype in this rabbit model was confirmed at 1-month AE by increased fluorescein ($P < 0.0001$), rose Bengal ($P < 0.0001$) staining, and elevated biomarker mRNAs (IL-1β, $P = 0.0027$; TNF-α, $P = 0.0045$; MMP-9, $P = 0.0295$). However, from 1-month on, fluorescein, rose Bengal staining and biomarkers reduced over time to near baseline (BE) at 4-months. Improvement of dry eye phenotype led to increased Schirmers test scores at 2 and 3-months ($P = 0.0009$) and reduced to baseline at 4-months AE. Conjunctival CFTR, NKA, NKCC1 and ENaCα did not show any up regulation over 4-months. AQP4 was up regulated in 2-months AE and declined to baseline at 4-months AE. AQP5 too was up regulated at 1-month AE and stayed up-regulated at 4-months AE.
Conclusions: In absence of LG, HG and NM a spontaneous resolution of dry eye phenotype was observed in our rabbit model. The conjunctival AQPs are possibly involved in a compensatory tear fluid balance at ocular surface. Similar role played by accessory lacrimal glands cannot be excluded.
Commercial Relationships: Dhruva Bhattacharya, None; **Yuan Ning**, None; **Fangkun Zhao**, None; **Rongji Chen**, None; **Jinsong Zhang**, None; **Mingwu Wang**, None

Program Number: 321 **Poster Board Number:** C0206
Presentation Time: 8:30 AM–10:15 AM
Nanowafer Drug Delivery for Restoration of Healthy Ocular Surface in Dry Eye Condition
Crystal Shin, Daniela Marcano, Johanna Henriksson, Ghanashyam Acharya, Stephen C. Pflugfelder. Ophthalmology, Baylor College of Medicine, Houston, TX.
Purpose: Dry eye is a steroid responsive ocular surface inflammatory disease. Therapeutic efficacy of dexamethasone treatment with a controlled release nanowafer (Dex-NW) or drops was compared in a murine desiccating stress model of dry eye.
Methods: Carboxymethyl cellulose (CMC) nanowafers and phospho dexamethasone loaded CMC nanowafers (Dex-NW) were fabricated by hydrogel template strategy. The in vivo efficacy of the Dex-NW was evaluated in the experimental dry eye induced mouse model by measuring corneal barrier function to 70kDa Oregon green dextran (OGD) and expression of inflammatory genes by RT-PCR.
Results: The nanowafer drug delivery system was designed for sustained controlled delivery of dexamethasone to ocular surface tissues (cornea/conjunctiva) in a controlled fashion and in vivo efficacy was demonstrated in a 5-day experimental dry eye induced mouse model. Corneal epithelial barrier disruption was measured by intensity of staining with fluorescent Oregon green conjugated dextran (OGD). Dex-NW placed on the bulbar conjunctiva on days 1 and 3 was equally effective as dexamethasone drops instilled twice a day eye for five days (Figure 1). RT-PCR analysis revealed that the down regulation of drug target genes IL-1α, IL-1β, TNF-α, IFN-δ, and MMP-3, and MMP-9 by Dex-NW treatment was comparable to twice a day topically administrated dexamethasone eye drop formulation (Figure 2). In both these studies, the dexamethasone delivered by the eye drop treatment was 20 μg compared to 5 μg of the drug delivered by the nanowafer during the same treatment period.
Conclusions: The Dex-NW was equally effective in suppressing dry eye induced corneal inflammation as conventional dexamethasone eye drops, even at a 4-fold lower drug concentration and alternate day dosing frequency.